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The Role of Tumor and Tumor Microenvironment on Breast Cancer-Associated
Adipocyte Plasticity

A dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy at Virginia Commonwealth University.

by

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February 4, 2019

Dedication

This dissertation is dedicated to my parents, Kestutis and Lina Vaitkus, to my sister, Katrina Vaitkus, and to my husband, William Pearce.

Thank you for providing me with the opportunities and support that undoubtedly guided me to where I am today, for your unwavering love and encouragement as I develop and pursue my dreams, and for your individual efforts that continue to help me along the way. I am incredibly fortunate and forever grateful. I love you all.

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List of Abbreviations

°C	Degrees Celsius
°F	Degrees Fahrenheit
2.4g2	Antibody recognizing the stalk region of murine CD23
AEE	Activity energy expenditure
APC	Allophycocyanin
B-Myb	Myb (myeloblastosis)-related protein B
β3	Beta-3
BAT	Brown adipose tissue
BEE	Basal energy expenditure
BMI	Body mass index
BMP	Bone morphogenetic protein (e.g. BMP2, BMP4, BMP7)
bp	Base pairs
BSA	Bovine serum albumin
BUV	Brilliant ultra violet
C/EBP	CCAAT-enhancer-binding protein (e.g. C/EBPα, C/EBPβ)
CAC	Cancer-associated cachexia
CD	Cluster of differentiation (e.g. CD11b, CD126, CD130)
cDNA	Complementary deoxyribonucleic acid
CFD	Complement factor D
CIDEA	Cell death-inducing DNA fragmentation factor, alpha subunit-like effector A

CLIA	Clinical Laboratory Improvement Amendments
CO ₂	Carbon dioxide
Cq	Quantitation cycle
CRSU	Clinical Research Service Unit
CT	Computed tomography
Cy7	Cyanine7
DAB	3,3'-Diaminobenzidine
DIT	Diet-induced thermogenesis
DLK1	Delta like non-canonical Notch ligand 1
DMSO	Dimethyl sulfoxide
EAR2	Nuclear receptor subfamily 2 group F member 6
EDTA	Ethylenediaminetetraacetic acid
EE	Energy expenditure
ER	Estrogen receptor
ELISA	Enzyme-linked immunosorbent assay
F4/80	Adhesion G protein-coupled receptor E1
FACS	Fluorescence active cell sorting
FASN	Fatty acid synthase
FBS	Fetal bovine serum
FcεR	Fc epsilon receptor
FDA	Federal Drug Administration
FDG PET/CT	¹⁸ F-fluorodeoxyglucose positron emission tomography/computed tomography

FGF21	Fibroblast growth factor 21
FH	Fumarate hydratase
FITC	Fluorescein isothiocyanate
GAD65	Glutamate decarboxylase 65
GP130	Glycoprotein 130
H&E	Hematoxylin and eosin
H ₂ O	Water
HER2	Human epidermal growth factor receptor 2
IACUC	Institutional Animal Care and Use Committee
IBMX	3-isobutyl-1-methylxanthine
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL6	Interleukin 6
IL6RA	Interleukin 6 receptor alpha
IL6ST	Interleukin 6 signal transducer
INFα	Interferon alpha
IRB	Institutional Review Board
JAK	Janus kinase (e.g. JAK1)
LDS	Lithium dodecyl sulfate
LEP	Leptin
LIPE	Hormone sensitive lipase
LLC	Lewis lung carcinoma
LPL	Lipoprotein lipase

Ly6	Lymphocyte antigen 6
MACS	Magnetic cell sorting
MAPK	Mitogen-activated protein kinase
MFI	Mean fluorescent intensity
MGLL	Monoglyceride lipase
mIWPA	Mouse immortalized white preadipocytes
MMTV	Mouse mammary tumor virus
MRN	Medical record number
MuRF1	E3 ubiquitin-protein ligase TRIM63
MYF5	Myogenic factor 5
NCBI	National Center for Biotechnology Information
NonidetP-40	Nonionic, non-denaturing detergent
O ₂	Oxygen
OPLAH	5-oxoprolinase
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Tween 20
PCR	Polymerase chain reaction
PDK4	[Pyruvate dehydrogenase (acetyl-transferring)] kinase isozyme 4, mitochondrial
PE	Phycoerythrin
PET (PT)	Positron emission tomography
PI3K	Phosphoinositide 3-kinase
PMA	Phorbol 12-myristate-13-acetate

PPAR	Peroxisome proliferator-activated receptor
PPARG	Peroxisome proliferator-activated receptor gamma
PPARGC1A	Peroxisome proliferator-activated receptor gamma coactivator 1 alpha
PR	Progesterone receptor
PRDM16	PR domain containing 16
PRMC	Protocol Review and Monitoring Committee
PTHrp	Parathyroid hormone related peptide
PTK2B	Protein tyrosine kinase 2 beta
PVDF	Polyvinylidene difluoride
PyMT (PyVT)	Polyoma Virus middle T antigen
qPCR	Quantitative polymerase chain reaction
RAG1	Recombination activating gene 1
rcf (g)	Relative centrifugal force (g-force)
REE	Resting energy expenditure
RER	Respiratory exchange ratio
RNA	Ribonucleic acid
rpm	Rotations per minute
SDS	Sodium dodecyl sulfate
SDHB	Succinate dehydrogenase subunit B
Ser727	Serine 727 residue
SLC27A1	Long-chain fatty acid transport protein 1
SLC29A1	Equilibrative nucleoside transporter 1

SOCS	Suppressor of cytokine signaling (e.g. SOCS3)
STAT	Signal transducer and activator of transcription (e.g. STAT3)
SV40	Simian vacuolating virus 40
SVF	Stromal vascular fraction
T3	Triiodothyronine
TBP	TATA box binding protein
TBS	Tris buffered saline
TBST	Tris buffered saline with Tween 20
TBX1	T-box transcription factor TBX1
TDAAC	Tissue and Data Acquisition and Analysis Core
TEE	Total energy expenditure
TME	Tumor microenvironment
TMEM26	Transmembrane protein 26
TNFRSF9	Tumor necrosis factor receptor superfamily member 9
TNM	Tumor, lymph node, metastasis – for cancer staging
Tris	Tris(hydroxymethyl) aminomethane
Tyr705	Tyrosine 705 residue
UCP	Uncoupling protein (1, 2, 3)
VCU	Virginia Commonwealth University
VCUHS	Virginia Commonwealth University Health System
WAT	White adipose tissue
Wnt	Wingless/Integrated
WT	Wild type

Abstract

THE ROLE OF TUMOR AND TUMOR MICROENVIRONMENT ON BREAST CANCER-ASSOCIATED ADIPOCYTE PLASTICITY

By Janina Vaitkus Pearce, B.S.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Clinical and Translational Research, Cancer and Molecular Medicine at Virginia Commonwealth University.

Virginia Commonwealth University, 2019

Director: Francesco S. Celi
Professor, Department of Internal Medicine
Division Chair, Division of Endocrinology, Diabetes, and Metabolism

Cancer-associated cachexia is a condition defined by a sustained net-negative energy imbalance. Although the different types of adipose tissue – white, beige, and brown – have been implicated in contributing to cancer-associated cachexia, the mechanisms of these maladaptive changes and their impact on whole-body energy expenditure have not been fully elucidated. Using breast cancer as our model, we demonstrate white adipose tissue browning in murine and human breast cancer; furthermore, we demonstrate that this effect is extremely localized and takes place early in tumor progression. We utilized *in vitro* cell culture techniques and demonstrate that cancer secreted factors and cross-talk with white adipocytes decrease expression of classic

white adipose tissue-related genes. We also demonstrate in murine and human culture models that cancer secreted factors reduce white adipocyte lipid droplet size, and cross-talk between cancer cells and adipocytes results in an increase in lipolysis-related gene expression. Interestingly, our results strongly suggest that in mice, neither cancer secreted factors nor cross talk with adipocytes can induce white adipose tissue browning, indicate that this process likely occurs independently of direct cancer interactions with local white adipocytes. We demonstrate that interleukin 6, a cytokine with previous implications in white adipose tissue browning, induces interleukin 6-mediated signaling; however, that signaling alone is not enough to directly induce white adipose tissue browning. We present preliminary data suggesting that immune cell population shifts within the white adipose tissue of mice with breast cancer tumors may be source of white adipose tissue browning. We show that the Virginia Commonwealth University Health System has an identifiable population of patients with cancer with what we hypothesize as *maladaptive* thermogenic adipose tissue activity, and discuss ongoing experiments aimed at understanding the implications of these changes on whole body energy expenditure in human patients. Lastly, in a case of autoimmune diabetes mellitus in the setting of an extra-adrenal paraganglioma, we demonstrate that the interaction between cancer and whole-body metabolism is multifaceted. Together, these experiments demonstrate that adipose tissue plasticity occurs in breast cancer (and other cancers), and that different drivers for individual changes exist within the tumor microenvironment. We predict that further exploration of the exact mechanisms and translational implications will provide useful information to lead to new therapeutic treatments for patients with cancer-associated cachexia.

Chapter 1: Background

Please note that some of the material within this Chapter comes from our previously published article, DOI 10.1177/1535370216683282. The final, definitive version of this paper has been published in Exp Biol Med (Maywood), Volume 242/Issue 5, March 2017 published by SAGE Publishing [1].

1.1 Energy balance

The First Law of Thermodynamics, also referred to as the Law of Conservation of Energy, states that energy cannot be created or destroyed [2]. In the context of metabolism, energy balance is defined as the difference between energy intake and energy expenditure (EE). Taken together with the Law of Conservation of Energy, changes in an individual's weight must be attributed to an imbalance in either energy intake or expenditure. A positive energy balance, where energy intake is greater than energy expended, causes weight gain; a negative energy balance, where energy intake is less than energy expended, results in weight loss (Figure 1). However, this balance is more complex than simply dietary intake and exercise; the classic “static,” or “linear,” model of energy balance is being replaced by the concept of “dynamic” energy balance, in which factors such as genetics, early life events, mood, stress, and metabolic and hormonal signaling also contribute to the regulation of an individual's energy balance [3].

In present-day society, the effects of sustained positive energy balance are seen in the global obesity pandemic, where rates of obesity have roughly tripled in the past 35 years [4]. While some individuals with obesity may be classified as ‘metabolically

healthy' [5], obesity is often associated with other health problems and increases the risk for development of comorbidities such as diabetes, cardiac disease, liver disease, and reproductive dysfunction, and cancer [6]. Obesity and its associated health problems also come with a substantial economic cost: in 2008 in the United States alone, the costs associated with obesity and its associated health problems were estimated to be about \$147 billion [7].

Deleterious effects can also be seen in cases of sustained negative energy balance. Severe burns, trauma, and sepsis can all result in a negative energy balance that causes undesirable health consequences [8-10]. While obesity increases the risk of cancer development [6], sustained negative energy balance in the context of cancer can result in cancer-associated cachexia (CAC), discussed in detail in section 1.4.

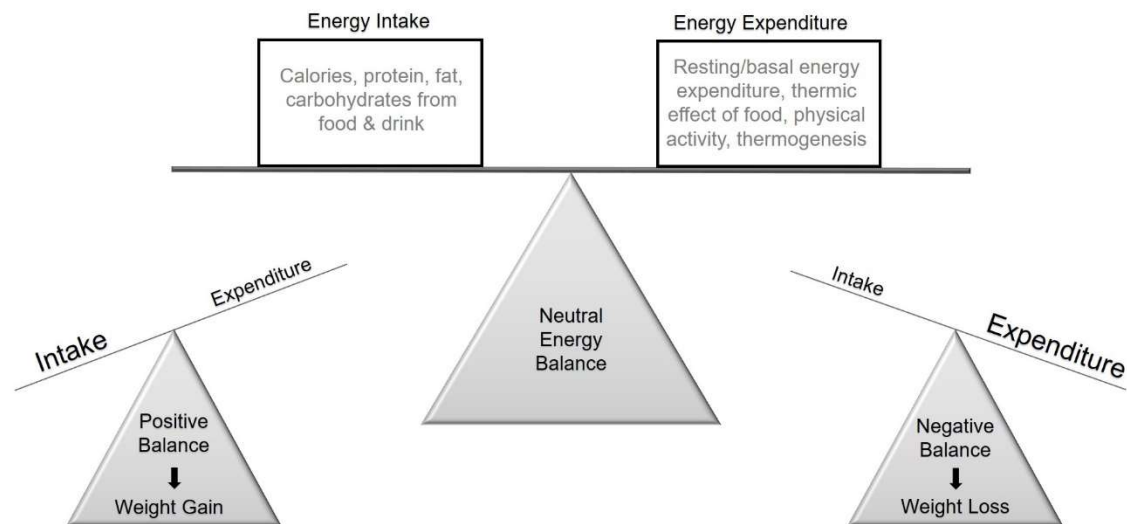


Figure 1. Conceptual overview of energy balance

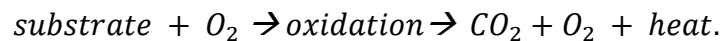
Energy balance is a complex, dynamic process with multiple contributing factors. Imbalances result in weight gain (positive balance, on left) or weight loss (negative balance, on right).

1.2 Measuring energy expenditure

Collectively, total energy expenditure (TEE) represents the total amount of energy used in daily functioning. TEE is comprised of resting energy expenditure (REE, also known as basal energy expenditure or BEE), activity energy expenditure (AEE), and thermic effects of food, or diet-induced thermogenesis (DIT). REE is the energy required to maintain basic cell and organ functions such as respiration and regulation of body temperature [11]. AEE is the energy used to perform exercise and non-exercise related tasks [12], and DIT is the energy used to metabolize substrates consumed in the diet [11]. Similar to energy balance, TEE is influenced by a large range of factors, including but not limited to age, body composition, thyroid hormone, catecholamines, pharmacologic therapies, and diseases [12].

Measuring energy expenditure (EE) is a useful tool in animal and human studies and can provide a point-estimate of energy utilization at rest, after eating, during exercise, and under other experimental conditions. Predictive equations, such as the Harris-Benedict equation, are historically based on specific patient populations. This is problematic, as our population's phenotype is changing over time and therefore invalidates many of these older predictive equations [11, 12].

There are several approaches to measuring EE. Heat, carbon dioxide, and oxygen are products of substrate utilization:



Direct calorimetry is a technique used to calculate EE by directly measuring heat loss [13, 14]. Subjects enter a sealed chamber to measure body heat production, and software is then used to calculate EE. While this can provide accurate measurements, it

is burdened with several limitations, the most significant that subjects cannot bring any devices that will increase heat production and confound results [11, 14]. Indirect calorimetry can overcome these disadvantages.

Indirect calorimetry is a technique that utilizes the other products of substrate utilization – by measuring CO₂ and O₂ gas exchange – to indirectly measure EE [13]. There are several different methods by which indirect calorimetry can be performed. Portable methods, which include a mouthpiece and mask attached to a small backpack-encased analyzer, allow for subjects to maintain relatively unrestricted mobility; however, they may be uncomfortable for the patient and limit the length of study design due to battery life constraints [14]. Metabolic carts using facemasks, mouthpieces, or ‘domed hoods’ are not limited by battery life and are fast and accurate in data collection; however, these are often uncomfortable for patients and limit patient mobility [14].

Whole-room indirect calorimeters – also known as “respiration chambers” or “metabolic chambers,” allow for maximum patient mobility and comfort. These are rooms that vary in size from small (fitting a twin sized bed or exercise bike, for example) to large (fitting a bed, toilet, sink, and exercise equipment simultaneously), providing the capability for a range of experimental designs (Figure 2 shows the VCUHS small and large open-circuit whole-room indirect calorimeters). While these large volumes can increase the equilibration time, our lab has demonstrated robust temporal accuracy in these systems [15]. Sleep studies, exercise studies, resting EE studies, combinations of these, and more are possible using these systems. Additional procedures such as blood and urine collection can be added to protocols using the whole-room indirect

calorimeters, and additional equipment can be used within the calorimeters to acquire additional data, such as accelerometers to measure movement.

A**B**

Figure 2. Whole-room indirect calorimeters at VCUHS

(A) Small and (B) Large whole-room indirect calorimeters located in the Clinical Research Service Unit (CRSU) in North Hospital at the Virginia Commonwealth University Health System (VCUHS).

1.3 Adipose tissue

Adipose tissue, previously viewed as an inert fat depot in isolated areas within the body, is now regarded as a large, interactive, multi-compartment organ with clear organization and anatomy [16-18]. Additionally, the secretomes of these depots can act locally (paracrine organ function) and systemically (endocrine organ function) [19, 20].

The composition of adipose tissue includes mature adipocytes, as well as other cell types that are important for each adipose tissue depots' function. These include vascular endothelial cells which provide the necessary vascular supply to the tissue, nerve endings from the sympathetic nervous system, adipocyte precursor cells, and a variety of immune cells [21-23].

Mature adipocytes are terminally differentiated cells, although recent evidence suggests that they can be reprogrammed [24]. Turnover of adipocytes is quite low, but de novo differentiation does occur [25].

1.3.1 White adipose tissue (WAT)

The largest component of adipose tissue is white adipose tissue (WAT), which primarily consists of large, spherical adipocytes with a unilocular lipid droplet consuming most of the cell volume [18]. WAT is found in both subcutaneous and visceral depots, and increased visceral WAT mass is associated with increased metabolic risk [26, 27]. WAT has important endocrine and paracrine roles throughout the body [18, 28, 29], and in general terms, functions to store energy, mainly in the form of triglycerides [30, 31]. Developmentally, white adipocytes are derived from myogenic factor 5 (MYF5)-negative precursors (Figure 3).

1.3.2 *Brown adipose tissue (BAT)*

In contrast to WAT, brown adipose tissue (BAT) primarily functions to expend energy [30]. Until recently, functionally-significant BAT was thought to be present only in neonates, undergoing rapid involution with age [30, 32-35], despite early reports that indicated the presence of BAT in adult humans [33, 34, 36]. The development and use of positron-emission tomography (PET) allowed for visualization of BAT in adult humans [37], and in combination with other functional analyses [38, 39], sparked the recent resurgence in interest in BAT's function in health and disease. Details of WAT and BAT development and gene signature are discussed elsewhere [30, 40, 41], but it is important to note that, at least in mice, anatomically-defined BAT adipocytes are derived from a cell lineage different from WAT, which instead share a lineage with myocytes (muscle cells) which are MYF5-positive [42, 43]. Adult human BAT depots are located near the aorta and within the supraclavicular region of the neck [18]. These adipocytes contain multilocular lipid droplets, and their ability to expend uniquely great amounts of energy is largely due to the presence and activation of a proton leakage pathway [44] mediated by uncoupling protein 1 (UCP1) – the hallmark of BAT function [33, 45].

UCP1 uncouples oxidative phosphorylation from ATP synthesis in the inner mitochondrial membrane (Figure 4) to dissipate energy in the form of heat [46, 47]. UCP1 is one of several members of the uncoupling protein family; of these, UCP2 and UCP3 are most similar to UCP1 in homology. UCP1 is found specifically in thermogenic adipocytes, UCP2 is expressed in many cell types [48], and UCP3 is expressed more selectively, most notably in skeletal muscle [49]. Both UCP2 and UCP3 are important in several cellular processes, for example, in regulating the cell's response to reactive

oxygen species-mediated oxidative stress and in fatty acid metabolism (reviewed by [48, 50, 51]; however, interestingly, of the uncoupling protein family, only UCP1 is able to confer a thermogenic phenotype – neither UCP2 nor UCP3 are capable of eliciting this effect [52, 53]. The *UCP1* gene in humans is located on chromosome 4 and has two regulatory elements in the 5' non-coding region that regulate its transcription. These bind to key transcriptional regulators such as peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PPARGC1A) and PR domain zinc finger protein 16 (PRDM16) (reviewed by [54]). UCP1 is regulated post-translationally as well. Free long chain fatty acids and purine nucleotides have been shown to serve opposing functions: purine nucleotides inhibit UCP1 activity, and free long chain fatty acids removing that purine-mediated inhibition to activate UCP1 function [55].

1.3.3 *Beige adipose tissue*

More recently, an additional type of adipocyte, defined as 'brite' (brown-in-white) [56] or 'beige' [57], was identified. These adipocytes are located within and share cell lineage with white adipocytes [30] but express UCP1 and function like brown adipocytes [56] (Figure 3A). While some authors suggest that beige cells develop *de novo* [58], the model of transdifferentiation – development of beige adipocytes from pre-existing white adipocytes [59] – predominates [18]. Interestingly, in addition to sustained exposure to cold, multiple different pathways promote the white-to-beige transition leading to a similar cellular phenotype, reviewed by Giralt [30]. Importantly, beige adipose tissue can expand in response to several mediators and is a target of both endocrine and paracrine stimuli [60].

This relatively 'new' beige adipose tissue has generated a great deal of excitement, as its function is similar to classical BAT in expending energy and may have additional protective roles against obesity [30]. On the other hand, its capability of developing, expanding, and activating under local stimuli, including extracellular matrix components [61], make this tissue a potential driver of CAC in response to tumor microenvironment.

Together, all three adipose tissue types (Figure 3A), as well as their differentiation and maintenance (Figure 3B), are clearly important in the energy balance that is disrupted in CAC.

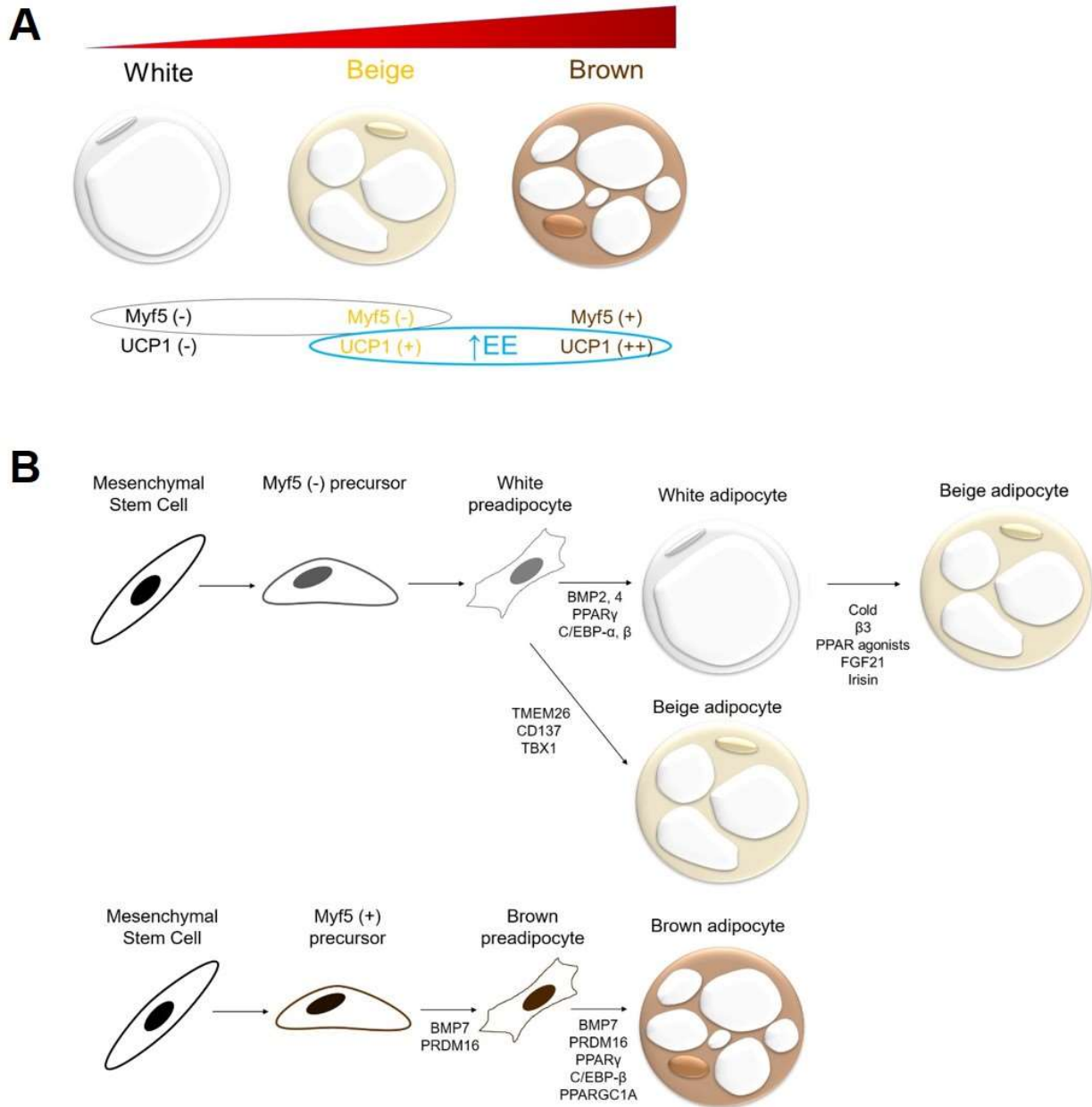


Figure 3. White, beige, and brown adipose tissue

(A) White adipose tissue ('White' in image) has the lowest thermogenic activity, while brown adipose tissue ('Brown' in image) has the highest. White and beige adipocytes share the same Myf5-negative lineage, but beige and brown adipose tissue share increased UCP1 expression which confers thermogenic activity. (B) General overview of process of adipogenesis and transdifferentiation of white to beige adipocytes ("WAT browning"). Abbreviations defined in List of Abbreviations.

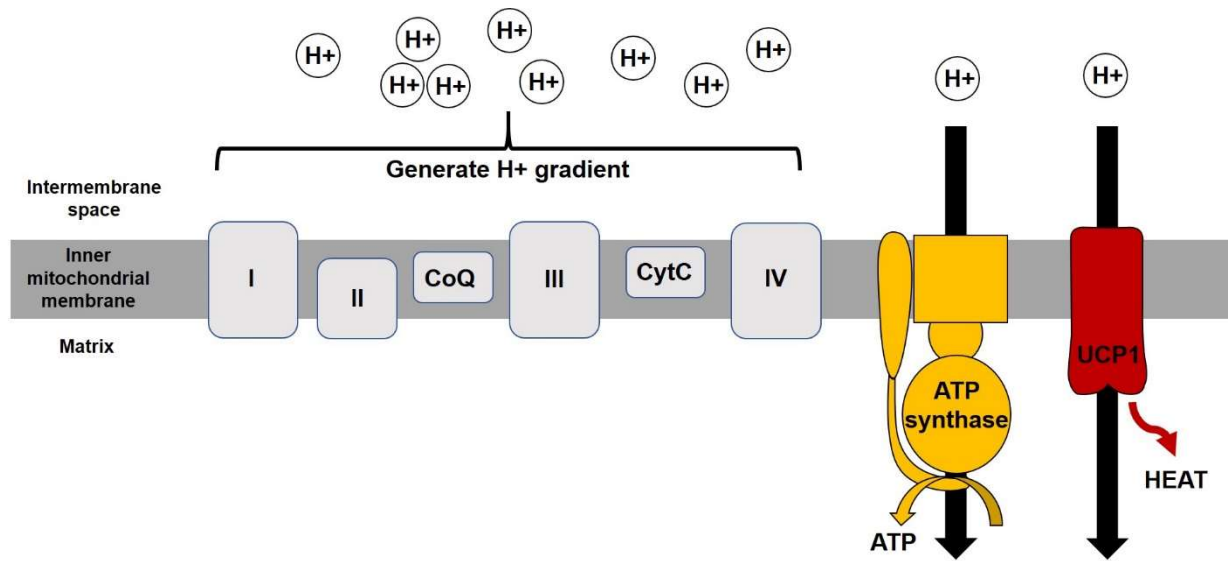


Figure 4. Uncoupling protein 1 (UCP1) is the hallmark of thermogenic adipose tissue

UCP1 is located within the inner mitochondrial membrane. Normally, protons generated by the electron transport chain (complexes I through IV as indicated in image) move through the ATP synthase to generate ATP; however, when UCP1 is activated, the proton gradient is collapsed via proton transport through UCP1, which generates heat.

1.4 Cancer-associated cachexia (CAC)

1.4.1 History, definition, and staging

Changes in metabolism and altered energy requirements have been documented in patients with cancer as early as the 1950s [62], yet it was not until the 1970s that the multifaceted nature of cancer-associated cachexia (CAC, also referred to as cancer cachexia) was appreciated [63, 64]. While early studies suggested that CAC was the result of tumor-driven anorexia alone [65], it is now established that CAC is not solely the result of decreased food intake; other contributing factors such as malabsorption and altered metabolism play a role as well [63, 64]. In the early 2000s, increased emphasis was placed on consolidating clinical and basic research findings to develop guidelines for defining, staging, and treating CAC.

CAC, according to a recent international consensus developed by Fearon and Strasser, is defined as a *“multifactorial syndrome defined by an ongoing loss of skeletal muscle mass (with or without loss of fat mass)”* [66]. Almost all cancers can be associated with CAC, although some more commonly than others [67]. Classically, CAC is described in three stages: precachexia, cachexia, and refractory cachexia. Several assessment criteria were proposed and described in detail by Fearon [66]; the most obvious is the degree of weight loss a patient experiences, but others include catabolic drive and loss of muscle mass and strength. The consensus guidelines were quickly applied to and validated in clinical practice, and additional assessment and management algorithms were proposed [68, 69].

While these guidelines represent significant improvements, there is still a lack of simple clinical indicators and markers to allow for earlier and straightforward

classification of CAC [69]. Despite increased awareness of this condition, CAC is still very common and treatments are inadequate [70], suggesting that a comprehensive understanding of the molecular and physiologic pathways leading to the development and progression of CAC is still lacking. The large inter-individual variability in the prevalence and severity of CAC in patients with the same tumor type [71, 72] further supports the need for improving the knowledge of the pathophysiologic mechanisms responsible for CAC. Given the high prevalence of obesity in the general population, early stages of CAC may be missed when the diagnosis is based purely on weight loss criteria (referred to as the 'obesity paradox' [72]). This factor demonstrates the need for improvement in the early diagnosis of this condition.

1.4.2 Prognosis, effects, and treatment options

The inverse association between weight loss and survival in patients with cancer is well known [73]. Recent estimates propose that CAC affects 60-80% of all patients with advanced cancer [69], and is directly implicated in at least 20% of cancer-related deaths [74], demonstrating that CAC is an independent risk factor for poor prognosis. The loss of body mass and altered body composition in CAC leaves patients vulnerable to increased toxicity from anti-tumor therapies such as chemotherapy [69, 75, 76]. This may directly result in increased morbidity and mortality, and may indirectly affect morbidity and mortality as the increased toxicity requires clinical treatment delays and dose reductions in therapy. Not surprisingly, patients with CAC additionally experience loss of physical function and decreased quality of life [69, 75].

Clinical advancements in defining and staging CAC have not been matched by similar improvements in treatment options and patient outcomes. Some studies suggest that CAC may be reversible if caught in the precachexia or cachexia stages [68, 76]. The main therapeutic strategies utilized in these stages include exercise, intense nutritional support, and removing any direct tumor causes (such as obstruction causing malabsorption) [68, 69]. Unfortunately, many cases are clinically diagnosed in the refractory cachexia stage, when CAC is often irreversible [66, 68] and nutritional support, current drug therapies, and other measures are ineffective in restoring a net-neutral or positive energy balance [77, 78]. In the past, many studies investigating therapeutic drugs to combat CAC, such as eicosapentaenoic acid diester [79] and bortezomib [80] have failed to demonstrate favorable outcomes. While experiments and studies investigating new potential therapeutic targets have been ongoing, when Fearon published his article in 2011, there were no active clinical drug trials for CAC [66]; since then, several drugs in the pipeline have entered into the clinical trial phases. A review by Dingemans and colleagues in 2014 identified 12 phase II clinical trials with 11 compounds [81]. Each of these drugs are expected to help combat CAC via one of the following mechanisms: increasing appetite, improving digestion, decreasing systemic inflammation, and improving the muscle synthesis-versus-degradation ratio [81]. Other drugs have recently entered phase III trials, but difficulties in meeting multiple clinical endpoints indicate the need for a more comprehensive approach. Anamorelin, a ghrelin-receptor agonist, demonstrated an ability to improve lean body mass in a phase II clinical trial with patients with non-small cell lung cancer and CAC [82]. However, this phase II trial, and a subsequent phase III trial, reported that while lean body mass

improved, functional improvements in hand grip strength did not [82, 83] – highlighting the potential discrepancy between proxies of end-organ effect and patient-centered significant outcomes. Enobosarm, a selective androgen receptor modulator, showed a significant increase in total lean body mass in phase II studies [84], but according to information thus far presented in abstract format, it has not yielded consistent endpoint results in phase III trials [85, 86]. As described below, there are many contributing factors to CAC, so the development of these drugs is certainly a step in the right direction. However, few compounds make it past phase II and III studies, and those that have (and will) may not fully address the spectrum of pathophysiologic components of CAC to be effective in mitigating or reversing all of the clinical components of CAC.

1.4.3 Contributing factors

The factors contributing to the development and maintenance of CAC are grounded in the axiom of energy balance: weight loss or gain only occurs when there is a sustained imbalance between energy intake and energy expenditure [64, 87]. In the case of CAC, there is a persistent net-negative energy balance, with components linked to both decreased energy intake and increased energy expenditure. Decreased energy intake may occur via several mechanisms, including anorexia caused by chemosensory distortions, malabsorption, and early satiety [66, 68, 71, 75]. Increased energy expenditure may result from inflammation, increased tumor metabolism, and altered/increased metabolism [68, 71, 72, 77]. To this end, the heterogeneity of adipose tissue is particularly relevant in the pathophysiology of CAC. Adipose tissue metabolism, aside from its more obvious role in obesity and diabetes, has been

demonstrated to play a role in other states of metabolic dysfunction such as CAC. As such, we identify and discuss the specific contributions of adipose tissue to the development and progression of CAC.

1.4.4 Adipose tissue contributions to CAC: lipolysis

The breakdown of adipose tissue – lipolysis – is perhaps the most evident component of adipose tissue's contribution to CAC [88]. Fat loss observed in patients with CAC is thought to occur via breakdown of adipose tissue (mainly WAT) in response to a negative energy balance due to cancer-associated anorexia and other pathologic factors recently reviewed by Ebadi and Mazurak [89, 90]. The importance of lipolysis in CAC was demonstrated in a study that showed that the inhibition of lipid mobilization can improve the CAC state [91]. A murine model of animals implanted with murine adenocarcinoma 16 tumors reported changes in adipose tissue that included shrunken adipocytes and decreased expression of adipose tissue transcription factors [92]. In 2018, investigators demonstrated early adipose tissue wasting in a new murine model of CAC in pancreatic cancer [93].

From a clinical perspective, a critical component of the body mass loss observed in CAC is the depletion of muscle mass, which usually precedes the observation of significant changes in adipose tissue mass and is associated with decrease in muscle function and mobility. Fearon and Strasser's consensus findings highlight that skeletal muscle loss is a necessity for a clinical diagnosis of CAC, but that adipose tissue loss may or not be present [66]. However, lipolysis and lipid wasting may occur to an extent before muscle loss [91]. Additionally, studies in a mouse model of colon cancer

demonstrated an increase in protein kinase-A mediated lipolysis in *early* stage cachexia [94], and this 'early' lipolysis was implicated in 1) the inception of a negative energy balance that worsens over the course of CAC progression, and 2) a direct loss of skeletal muscle [95]. Lipolysis results in increased free fatty acids in circulation, which then get taken up by skeletal muscle; the excess of intramuscular free fatty acids results in several biochemical changes, such as the expression of ubiquitin lipases Atrogin-1 and MuRF [96], that lead to skeletal muscle atrophy [97]. Indeed, Stephens showed a positive association between the extent of body weight loss in cancer patients and the amount of lipid droplet accumulation within skeletal muscle cells [98]. In a study of late-stage CAC, protein kinase-A mediated lipolysis was not observed as in early stages, but instead more lipases were observed and contributed to skeletal muscle dysfunction and atrophy [99]. These findings suggest that there are likely different stages of lipid metabolic responses in CAC. Physiologically, one would expect that lipolysis and resulting loss of WAT mass would stimulate other pathways in the body to drive anabolism and energy intake, such as leptin. Leptin is produced by WAT and its levels are positively correlated to a patient's state of adiposity, regardless of age or body mass index (BMI) [89]. Low levels of leptin are expected in patients exhibiting fat loss with CAC, which should result in increased activation of orexigenic pathways; however, studies demonstrate that this feedback may be disrupted in cancer, resulting in an undesirable decrease in signaling pathways such as neuropeptide Y [100]. It is important to recognize that inflammation is known to be a key player in lipolysis and the general increased catabolic drive observed in CAC (reviewed by Penet [77]). Since adipose tissue depots have interspersed lymphocytes [18] and macrophages [101],

there may be a functional relationship between lipolysis and increased inflammation. However, CAC can present even in the absence of frank systemic inflammation [66], and therapies targeting inflammatory cytokines such as tumor necrosis factor-alpha and eicosapentaenoic acid diester have not been successful in ameliorating CAC [79, 102]. This finding is consistent with the multifactorial nature of CAC.

1.4.5 Adipose tissue contributions to CAC: beige and brown adipose tissue expansion and activation

Several studies highlight increased resting energy expenditure in animal models as well in humans affected by CAC, and point to adipose tissue as a culprit [103-105]. While lipolysis literally represents a loss of adipose tissue (especially WAT) mass, BAT and beige adipose tissue depots appear to contribute to CAC via increased energy expenditure.

BAT was first proposed as a contributor to CAC in 1989 [106], but this hypothesis was not investigated further until the rediscovery of functional BAT in adult humans [38]. In a mechanistic study, Tsoli and colleagues found that activation of BAT (via increased *Ucp1* expression) contributed to the development of CAC in mice with cachectic colon cancer cell line injections [103]. Interestingly, CAC and BAT activation were not present in mice with non-cachectic colon cancer cell line injections [103]. In another study, BAT activity was measured via ^{18}F -fluorodeoxyglucose positron emission tomography/computed tomography (FDG PET/CT) in human patients with cancer, and was found to correlate positively with cancer stage [107]. However, classical BAT is relatively limited in mass, localized to relatively small depots within the body, therefore

severely limiting its capacity to offer substantial contributions to energy expenditure and CAC in humans [108, 109]. More studies are needed to provide a quantitative assessment of classical BAT's contributions to CAC [110].

WAT, however, has much larger depots, so the expansion of beige adipose tissue within WAT depots may confer greater increases in energy expenditure, and may contribute significantly to CAC. Not surprisingly, WAT browning was identified in patients with pheochromocytomas [111], neuroendocrine tumors that secrete norepinephrine, a known trigger of white-to-beige trans-differentiation. However, WAT browning has also been observed in other tumor models beyond catecholamine-secreting tumors. Mechanistic studies in mice include genetic models of Kras-lung, Kras-pancreatic, and K5-SOS carcinomas [112], chemically induced liver carcinoma [112], graft/injection models of B16 melanoma [112] and Lewis lung carcinoma [112, 113], C26 colon carcinoma with and without interleukin 6 (IL6) expression [112], and human pancreatic carcinoma xenografts [112]. In these models, investigators reported the presence of WAT browning and its contribution to the observed CAC. This was demonstrated by increased expression of *Ucp1* mRNA and UCP1 protein [112, 113], and also through increased expression of other genes crucial for beige adipose tissue development, such as *Prdm16* and *Ppargc1a* [113]. The authors also performed functional analyses of the proposed beige adipose tissue to demonstrate increased energy expenditure using oxygen consumption rate assays as well as body weight, tissue weight, physical activity, oxygen uptake (VO_2), carbon dioxide production (VCO_2), respiratory exchange ratio (RER), and heat generation measurements [112, 113]. Additionally, two drivers of white-to-beige trans-differentiation were identified: IL6 [112]

and parathyroid hormone-related peptide (PTH-rp) [113]. These are the first studies to identify causative agents secreted by tumors that contribute to WAT browning and result in increased energy expenditure, which contributes to CAC. importantly, WAT browning was observed early in the progression of CAC, before skeletal muscle atrophy occurred (consistent with the observations in lipolysis studies [91, 94]), suggesting that adipose tissue dysfunction may occur prior to clinically-evident adipose tissue loss, contributing to the development of CAC. Furthermore, Petruzzelli and colleagues examined, via immunohistochemistry, adipose tissue samples acquired from human patients with CAC and a multitude of cancers: Kaposi's sarcoma, melanoma, cholangiocarcinoma, colon adenocarcinoma, pancreatic neuroendocrine cancer, pleomorphic lung carcinoma, and lung adenocarcinoma [112]. Results showed that many of the WAT samples had increased UCP1 expression and adipocyte atrophy. While there is still much to understand, these results clearly support the role of WAT browning in the development and progression of CAC.

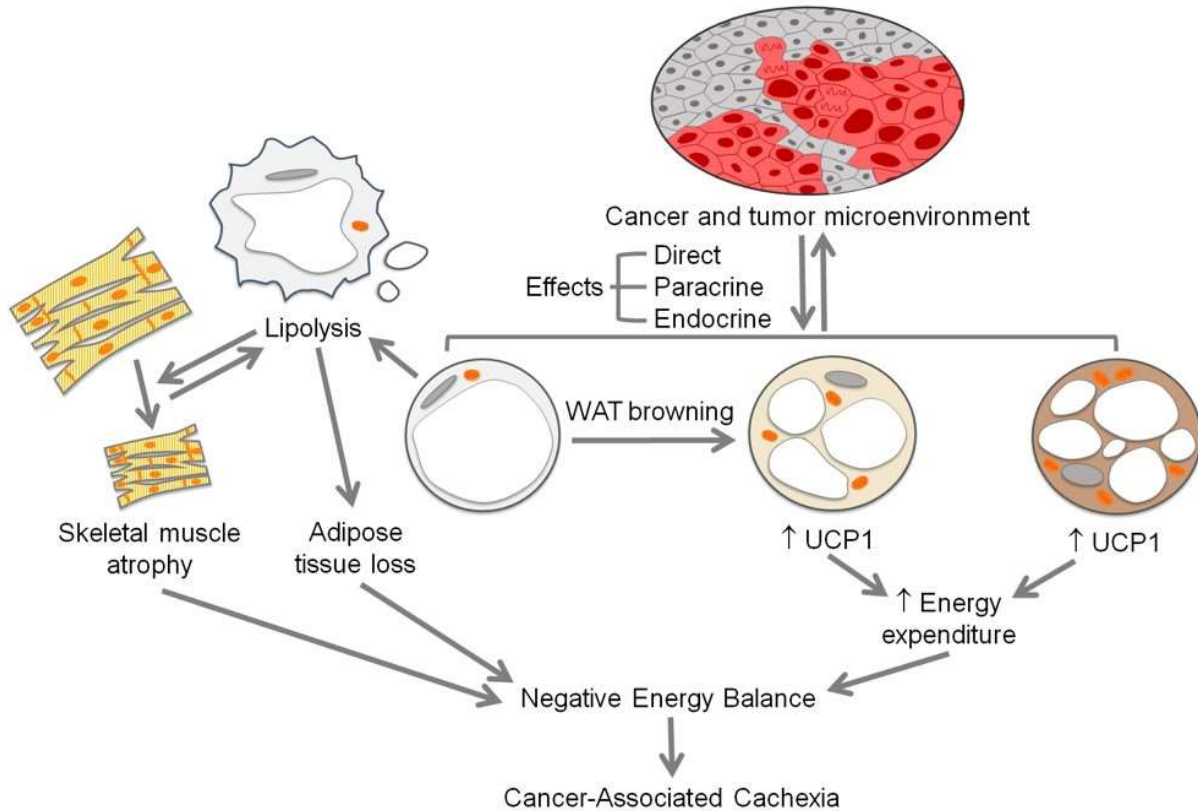


Figure 5. Adipose tissue in CAC

A summary of adipose tissue contributions to cancer-associated cachexia (CAC). Cancer and its microenvironment (top) influence white, beige, and brown adipose tissue (colored respectively). WAT undergoes lipolysis, which results in a direct loss of adipose tissue mass and also contributes to skeletal muscle mass loss. As the process progresses, skeletal muscle loss may act as positive feedback for further adipose tissue lipolysis. WAT may also undergo browning to undergo trans-differentiation to beige adipose tissue, expressing uncoupling protein 1 (UCP1) and thereby expending greater amounts of energy. Similarly, existing classical brown adipose tissue may be activated, resulting in greater UCP1 expression with a resulting increase in energy expenditure. Collectively, these changes result in a net-negative energy balance, which contributes to the development and progression of CAC.

1.4.6 The other side of the coin: the role of adipose tissue in promoting cancer progression

It is important to recognize that the adipose tissue-cancer interactions also go in the opposite direction. Cancer may directly and indirectly affect adipose tissue, and adipose tissue may directly and indirectly affect cancer growth and survival, as well [114]. Recent studies demonstrated that adipose-derived fibroblasts and cancer-associated adipocytes can contribute to cancer progression [19, 115, 116], and another study demonstrated that increased beige and brown adipose tissue contributes to tumor progression [117]. Since higher rates of lipolysis and browning are strongly correlated with later-stage CAC, the hypothesized cross-talk between tumor and adipose tissue may generate a positive feedback loop facilitating cancer progression and the clinical impact of CAC.

1.4.7 Potential therapeutic strategies to restore adipose tissue homeostasis in CAC

As mentioned previously, aberrations in adipose tissue metabolism and homeostasis in malignancy result in a decrease in both adipose tissue and muscle mass. An ideal therapeutic approach would improve both adipose tissue and lean muscle mass; however, this has not been successful due to the differences in cell lineage of muscle and WAT. Their common precursor is the mesenchymal stem cell, but adipogenesis and myogenesis have very different differentiation signals and pathways, such differences in Wnt signaling [118]. Differences are also observed mechanically, as softer substrates promote adipogenesis while stiffer substrates promote myogenesis [119]. These differences, and subsequent lack of overlapping triggers, make the

development of a single therapeutic aimed at increasing both WAT and muscle mass incredibly difficult.

This relationship between adipose tissue changes and muscle loss is observed in other non-malignancy states as well: in postmenopausal women with type 2 diabetes mellitus, for example, increased intramuscular adipose tissue was associated with decreased hand grip strength [120]. In the elderly population, increased thigh muscle lipid content, represented by lower skeletal muscle attenuation coefficients on computed tomography images, was demonstrated to be negatively associated with muscle attenuation and strength [121]. Even in patients without metabolic disease, increased thigh intramuscular adipose tissue is associated with predictors of increased metabolic syndrome risk [122]. Current clinical trials investigating potential therapeutic agents for patients with CAC are promising, but the drugs being tested do not directly address the contributions of white, beige, and brown adipose tissue to CAC (such as reducing the release of free fatty acids from WAT, or inhibiting the white-to-beige transition). AR-42, a histone deacetylase, has recently demonstrated preclinical success in reducing levels of MuRF1 and Atrogin-1 lipases and decreasing muscle degradation [123]. So, while still mostly speculative, it is possible that restoring adipose tissue homeostasis by targeting these areas of dysfunction will contribute to further improvements in patient-centered outcomes. For example, inhibition of WAT lipolysis and its downstream effects on skeletal muscle atrophy could lead to improvement of hand-grip strength (used as a proxy for overall muscle strength and function) and assessments of physical activity (usually via patient-reported rating of physical functioning). Patients' self-reported quality of life and psychosocial status will likely improve if adipose tissue mass loss (and

skeletal muscle, as well) was dampened – in part because patients may feel that they are in better control of their weight and can keep their strength, and also in part because conservation of adipose tissue mass would likely result in a decrease in anti-tumor therapy toxicities. While studies investigating a ketogenic diet demonstrate improved quality of life in patients with advanced cancer [124], to our current knowledge, no studies have investigated the capability to restore adipose tissue mass and the subsequent effect of restoring adipose tissue mass on CAC. A comprehensive understanding of the physiology and pathophysiology of adipose tissue, cancer, and CAC will hopefully lead to the discovery of key components and drivers within the development and progression of CAC that can be manipulated and targeted to benefit the patient. This is not a simple task, but as research progresses, the future applicability to patients suffering from CAC must always be considered a primary endpoint.

1.5 Breast cancer

1.5.1 Epidemiology and rationale for use

Changes in normal breast tissue can be malignant or benign. While benign conditions, such as fibroadenomas, fibrocystic changes of the breast, and cysts, are estimated to account for approximately 80% of breast conditions, malignant breast changes – breast cancer – have a staggering prevalence and impact on human health. Worldwide, breast cancer is the most common cancer type diagnosed, and the most common cause of cancer death in women [125]. Among women in the United States, breast cancer is the most common cancer diagnosed and the second-leading cause of cancer death [126, 127]. In the United States alone, an estimated 266,120 new cases of

breast cancer will have been diagnosed in women in 2018. About 20% of patients with breast cancer die from the disease, often due to incurable metastatic disease [126].

A 2009 study including over 2000 patients with breast cancer showed that approximately 25% of patients with breast cancer are diagnosed with cachexia [128]. Although the percentage of patients with CAC in other cancers was found to be greater, such as in gastric and esophageal cancer (with 41.5 and 41.9% of patients with CAC, respectively [128]), the enormous prevalence of breast cancer relative to these other cancers reinforces the importance of studying CAC and WAT browning within this cancer type. A tabular visualization of these numbers is included in Table 1.

While the high prevalence of breast cancer, both in the United States and world-wide, serves as justification for the clinical importance of studying CAC in breast cancer, there are advantages to utilizing breast cancer for mechanistic studies of WAT browning in cancer *in vitro* and *in vivo*. Breast cancer is usually immediately adjacent to breast adipose tissue based on basic breast anatomy, which allows for local, mechanistic studies. Additionally, because localized breast cancer and breast tissue does not immediately interact with other systems, using breast cancer as a model to study WAT browning in cancer eliminates the confounding variables seen in other models (such as malabsorption in gastrointestinal-related cancers).

Table 1. Burden of cancer-associated cachexia (CAC) in breast cancer

Cancer type	Estimated number of new patients (men and women) diagnosed with disease in 2018 [126]	Percentage of patients with any one of the cachexia definitions [128]	General breast cancer CAC burden (estimated new cases x percentage with cachexia)
Breast	268,670	24.8%	66,630
Gastric	26,240	41.5%	10,890
Esophageal	17,290	41.9%	7,245

1.5.2 Pathophysiology

The breasts, or mammary glands, comprise a specialized organ with specialized function in women: to produce milk to provide nutrients and passive immunity to their nursing offspring after childbirth. The glands' anterior border is comprised of the epidermis (skin) and superficial fascia of the anterior thoracic wall, and the posterior border consists of the deep fascia of the anterior thoracic wall, retromammary bursa, and pectoralis muscles. Each gland contains approximately of 10-20 lobes connected by ducts, ultimately forming the lactiferous sinus which then exits through the nipple anteriorly. The lobes are supported by fibrous stroma, Cooper ligaments, and adipose tissue. The tissue also contains well-organized vasculature, innervation, and lymphatic drainage (detailed in [129]).

Risk factors for the development and progression of breast cancer have been studied extensively, and include nulliparity, familial history, history of benign breast disease, and obesity [130, 131]. Considering that the ducts and lobules comprise the mammary glands, it is not surprising that the two main types of breast cancer are ductal and lobular. These cancers are further classified into subtypes based on several molecular markers: estrogen receptor (ER), progesterone receptor (PR); and human epidermal growth factor receptor 2 (HER2). The four main subtypes of breast cancer are: luminal A (ER and/or PR+, HER2-); luminal B (ER and/or PR+, HER2+); HER2-enriched (ER-, PR-, and HER2+), and triple negative (ER-, PR-, and HER2-). It is important to note that the presence of other receptors and markers, such as androgen receptor, Ki67, B-Myb, and Twist can be useful, and studies are ongoing to understand the predictive values of these more recent, novel markers [132].

Tumor size (T), lymph node spread and extent (N), and extent of metastases (M) are the three criterion that make up the TNM staging algorithm, which create a staging 'score' ranging from 0 (representing breast cancer in-situ) to IV (representing the most advanced breast cancer) [131]. Breast cancer most commonly metastasizes to the bone, lung, brain, and liver [133]. Treatment options for breast cancer are variable depending on the type and extent of disease. Surgery, radiation, and chemotherapy are all therapeutic options, and hormonal and targeted therapies can be used as well [131].

1.6 Tumor microenvironment (TME)

1.6.1 General concepts

The tumor microenvironment (TME) has several features that distinguish it from normal, healthy tissue. In addition to the presence of tumor (cancer) cells, the TME often has mesenchymal stem cells abnormally recruited as well as activation of cancer-associated fibroblasts. The TME is often characterized by necrosis, hypoxia, and an acidic pH, with increased interstitial pressure, abnormal extracellular matrix deposition, and abnormal, disorganized, and often 'leaky' vasculature with increased blood flow [134, 135]. This leaky vasculature facilitates increased infiltration of a variety of immune cells, many of which are implicated in cancer-related processes [136]. As described already in sections 1.4.4 through 1.4.6, changes in adipocytes and adipose tissue depots may also contribute to cancer progression.

In breast cancer, key changes within the TME are known to aid in tumor progression and metastasis [137]. Mitochondrial function (or dysfunction) in different TME compartments has been shown to contribute to breast cancer growth [138].

Altered signaling induced by breast cancer cells promotes tumor-beneficial processes such as angiogenesis, resistance to therapy, and cell proliferation [139]. The TME is associated with dynamic changes in inflammatory status, with many different chemokines and cytokines involved. Interleukin 6 (IL6) is one such cytokine.

1.6.2 Interleukin 6 (IL6)

IL6 is a glycosylated protein comprised of 184 amino acids, synthesized and secreted by a variety of cells, such as neutrophils, macrophages, smooth muscle cells, T cells, monocytes, fibroblasts, endothelial cells, and even preadipocytes [140-143].

There are three IL6-initiated signaling cascades: the janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway, the mitogen-activated protein kinase (MAPK) pathway, and the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) pathway (reviewed by [21]). We focus on the JAK/STAT pathway due to its implicated role in other models of WAT browning [144]. JAKs are constitutively associated with the cytoplasmic tail of GP130 [140]; after the IL6:IL6RA complex binds GP130, JAKs are then activated by autophosphorylation and function to phosphorylate tyrosine residues in the cytosolic portion of GP130. This recruits STAT transcription factors to propagate target effects, and suppressors of cytokine signaling (SOCS) which act as feedback inhibitors [145].

Signaling initiated by IL6 is a multi-step process. First, IL6 must bind to a specific receptor, IL6 receptor alpha (IL6RA; also known as IL6R, or cluster of differentiation CD126). IL6RA can be either membrane-bound or soluble; if soluble, it is usually the result of proteolytic cleavage from the membrane (although translation of alternatively

spliced mRNA may also be a source) [146-148]. IL6RA is *not* ubiquitously expressed; instead, it is cell-specific, primarily found on hepatocytes, neutrophils, monocytes, and T cells [149]. After IL6 binds to IL6RA, this IL6:IL6RA complex then associates with the transmembrane protein glycoprotein 130 (GP130; also known as IL6 signal transducer IL6ST, IL6RB, or cluster of differentiation CD130) [150]. IL6 alone will not bind to GP130 without first associating with IL6RA [151]. Unlike IL6RA, GP130 is expressed on all cells within the body [152, 153]. Dimerization of GP130 serves as the signal transducer for propagation of the JAK/STAT pathway [151].

IL6 is an inflammatory cytokine with pleiotropic actions, and has various roles in the breast and tumor environment, which includes cancer cells and adipocytes as well as adipocyte precursors, endothelial cells, and macrophages [154]. Elevated IL6 levels increase protein catabolism and skeletal muscle wasting which contributes to CAC [155]. Through its canonical signaling pathway via signal transducer and activator of transcription 3 (STAT3), the role of IL6 in CAC has been expanded to include other organ systems beyond skeletal muscle, including adipose tissue (reviewed by [156]). IL6 is thought to be involved in the dedifferentiation and inflammatory characteristics of breast cancer-associated adipocytes [157] and has been shown to stimulate WAT lipolysis [158]; however, its roles in BAT activation and WAT browning are only of recent interest. Increased BAT activity and WAT browning occurs in cancer and may contribute to CAC [103, 112, 113]. Specifically, IL6 has been implicated as a direct driver of WAT browning in models of colon cancer [112, 158].

1.7 Summary

Sustained net-negative energy imbalance, as seen in CAC, represents a substantial clinical burden. White, beige, and brown adipose tissues are known to be involved in the development and progression of CAC, although exact mechanisms are not fully understood for all processes, especially in breast cancer. Breast cancer represents an ideal model to study the interactions between tumor, tumor microenvironment (especially IL6), and white adipose tissue.

Chapter 2: Materials and Methods

All mice were bred and maintained in Virginia Commonwealth University animal facilities and utilized in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) at VCU: AM10309 (Dr. Xianjun (Frank) Fang laboratory), AD10000814 (Dr. Charles Clevenger laboratory), AD10001219 (Dr. Paula Bos laboratory), AM10065 (Dr. Daniel Conrad, now Dr. Rebecca Martin laboratory), AD10001447 (Dr. Francesco Celi laboratory).

All human subjects research was performed in accordance with VCU IRB and PRMC-approved protocols (HM20009089 and MCC-17-13470, respectively).

2.1 Tissue acquisition

2.1.1 Mouse tissue acquisition

In collaboration with Dr. Frank Fang, female B6.FVB-Tg(MMTV-PyVT)634Mul/LelJ mice (also referred to as “C57BL/6J MMTV-PyVT” mice, Jackson Laboratory, stock #022974) at approximately 4 months of age were euthanized via isofluorane and cervical dislocation before being transferred to our lab for tissue extraction. Bilateral adipose tissue pads (interscapular, axillary, inguinal, and ovarian) were harvested and used for immunohistochemistry (see section 2.2) and gene expression analysis (see section 2.14). For axillary and inguinal adipose tissue pads, when tumor was present, some of the adjacent tumor was included with the adipose tissue that was placed in formalin for downstream immunohistochemistry.

In collaboration with the lab of Dr. Charles Clevenger, female PyMT FVB/N-Tg (MMTV-PyMT) 634 Mul/J mice (also referred to as “FVB MMTV-PyMT” mice, Jackson Laboratory, stock #002374) at approximately 4 and 13 weeks of age, as well as age-matched female control FVB/NJ mice (Jackson Laboratory, stock #001800), were euthanized before transfer to our lab for tissue extraction. Bilateral adipose tissue pads (interscapular and inguinal) were harvested and used for gene expression and protein analyses (see sections 2.14 and 2.15, respectively).

C57BL/6J wild-type mice (Jackson Laboratory, stock #000664) and BALB/cJ wild-type mice (Jackson Laboratory, stock #000651) were euthanized at approximately 3 weeks of age via isoflurane. Inguinal white adipose tissue pads were removed under sterile conditions in a cell culture hood and used to isolate primary stromal vascular fraction cells for cell culture (see section 2.4.1).

2.1.2 Human tissue acquisition

Benign breast tissue from women undergoing elective breast reduction surgeries was obtained through the VCU Tissue and Data Acquisition and Analysis Core (TDAAC). Deidentified samples were then moved under the cell culture hood, fibrous and connective tissue was removed, and adipose tissue was used for immunohistochemistry (see section 2.2.2) and isolation of primary stromal vascular fraction cells for cell culture (see section 2.4.2).

Slides with deidentified human samples of breast cancer with adjacent adipose tissue were obtained from the TDAAC for use in immunohistochemistry.

2.2 Immunohistochemistry (IHC)

2.2.1 Mouse IHC

Bilateral adipose tissue pads (interscapular, axillary, inguinal, and ovarian), with tumor when applicable, were harvested from female B6.FVB-Tg(MMTV-PyVT)634Mul/LelJ mice at approximately 4 months of age and immediately placed into 10% buffered formalin (Fisher Chemical) for 6 days. Tissues were then transferred to 70% ethanol until embedding and sectioning was performed by the VCU Massey Cancer Center's Cancer Mouse Model Shared Resource. All tissues were embedded with paraffin using a standard dehydration, clearing, and paraffin infiltration program via a Sakura Tissue Tek automated processor, and were sectioned at 5µm thickness.

Mouse UCP1 expression in the tissues was detected via immunohistochemistry using the SuperPicture 3rd Gen IHC Detection Kit (Invitrogen 87-8973) reagents and slight protocol modifications. Slides were deparaffinized in xylene and rehydrated in a graded series of ethanol, ending with distilled water and finally PBST. Slides were then placed in citrate buffer (Sigma-Aldrich C9999) diluted to 1X in distilled water and heated in a microwave for antigen retrieval. Peroxidase quenching solution (included in kit) was placed on slides and incubated for 10 minutes. After washing with PBST, slides were covered with 5% BSA (Fisher Scientific BP1600-100, diluted in TBST) for one hour. The slides were incubated in UCP1 primary antibody (abcam 10983; diluted 1:500 in 1% BSA) or 1% BSA (to serve as post-antibody negative control) at 4°C in a dark cold room overnight. The next day, after washing with PBST, HRP Polymer Conjugate (included in kit) was added to cover the tissue area and left to incubate for 30 minutes. Slides were washed in PBST and DAB Chromogen (included in kit) was added for approximately 2

minutes to visualize binding. Slides were then washed with tap water and counterstained with diluted Hematoxylin Solution Gill No.2 (Sigma-Aldrich GHS232) for 30 seconds. After washing with tap water, slides were dehydrated using a reverse-graded series of ethanol, ending with xylene, and glass slides were mounted using HistoChoice Mounting Media (Amresco/VWR H157).

Slides were digitally scanned using the NanoZoomer 2.0-HT Whole Slide Imager Digital Pathology Slide Scanner (Meyer Instruments) by the Department of Pathology.

2.2.2 Human IHC

Slides were stained by the VCU Massey Cancer Center's Cancer Mouse Model Shared Resource Core for UCP1 using primary antibody (abcam ab155117, diluted 1:200). Stained slides were scanned at 20X magnification by the Core facility. Slide images were captured using Phenochart 1.0.9 software.

2.3 IHC staining intensity: qualitative and quantitative measures

Qualitative assessment of IHC intensity was determined via blinded scoring by Dr. Trang Le in the Division of Endocrinology, Diabetes, and Metabolism. Images were assigned random number labels and sent to Dr. Le for scoring. For each sample, Dr. Le assigned a numerical value to grade the average staining intensity in the adipose tissue at 1) the tumor-adipose tissue interface and 2) the adipose tissue furthest from the tumor. The following numerical scale was used: 0 = no staining; 1 = weak staining; 2 = moderate staining; 3 = strong staining. These values were used to calculate the gradient of staining intensity.

Quantitative assessment was performed by computation analysis. The IHC images were processed and analyzed in MATLAB 2017a. A customized algorithm was developed to automatically detect the brown pixels and count the number of brown pixels by columns of each IHC image. The counts were plotted against the column index (which indicates the distance to the tumor cells) for curve fitting.

2.4 Isolation of primary stromal vascular fraction (SVF) cells from adipose tissue

Stromal vascular fraction (SVF) isolation was performed according to established protocol [159] with slight modifications for both mouse and human white adipose tissue. See Figure 6 for visual representation of this process.

2.4.1 Mouse SVF

For each C57BL/6 or BALB/c mouse, left and right inguinal fat pads were homogenized and combined into one tube, then digested in 1mg/mL Collagenase A (Roche 10103586001) in sterile PBS at 37°C for 90 minutes with gentle inversion of tubes every 10-15 minutes. After digestion, the cell solution was filtered through a 100µm cell strainer to remove any large, undigested tissue. The remaining cell solution was centrifuged at 200g for 10 minutes at room temperature. Supernatant was removed (including floating lipids) to leave a pellet of SVF. This SVF pellet was resuspended in 5mL of basal cell culture medium consisting of DMEM/F12+Glutamax (Thermo Fisher 10565-018), 10% fetal bovine serum (FBS; Thermo Fisher 10082-147), 200U/mL penicillin, 0.2mg/mL streptomycin, and 25µg/mL amphotericin B (Sigma Aldrich A5955) and 100µg/mL Normacin (InvivoGen ant-nr-1, lot NOL-39-05A) and

plated on to a T25 tissue flask (Cellstar 690175). Cells were kept in a cell incubator (HERAcell 150i) at 37°C and 5% CO₂ from this point forward. Cell culture medium was changed after 4 hours to remove cells that had not adhered (especially red blood cells). Cell culture medium was changed the next day and then every 2 days until cells reached 90% confluency, at which point they were passaged with Trypsin (Gibco 25200-056) and expanded to T75s (Corning 430641U) or frozen in basal cell culture medium and 10% DMSO (Sigma D2650).

2.4.2 Human SVF

Adipose tissue was cut into small pieces and SVF pellet was obtained using methods described in section 2.4.1. The resulting human SVF pellet was resuspended in 5mL of basal cell culture medium consisting of DMEM/F12+Glutamax, 200U/mL penicillin, 0.2mg/mL streptomycin, and 25µg/mL amphotericin B, supplemented with either 1) 10% FBS or 2) components included in Fibroblast Growth Kit-Low serum (ATCC, PCS-201-041) and plated on to a T25 tissue flask. Cells were kept in a cell incubator and cared for using same methods described in section 2.4.1.

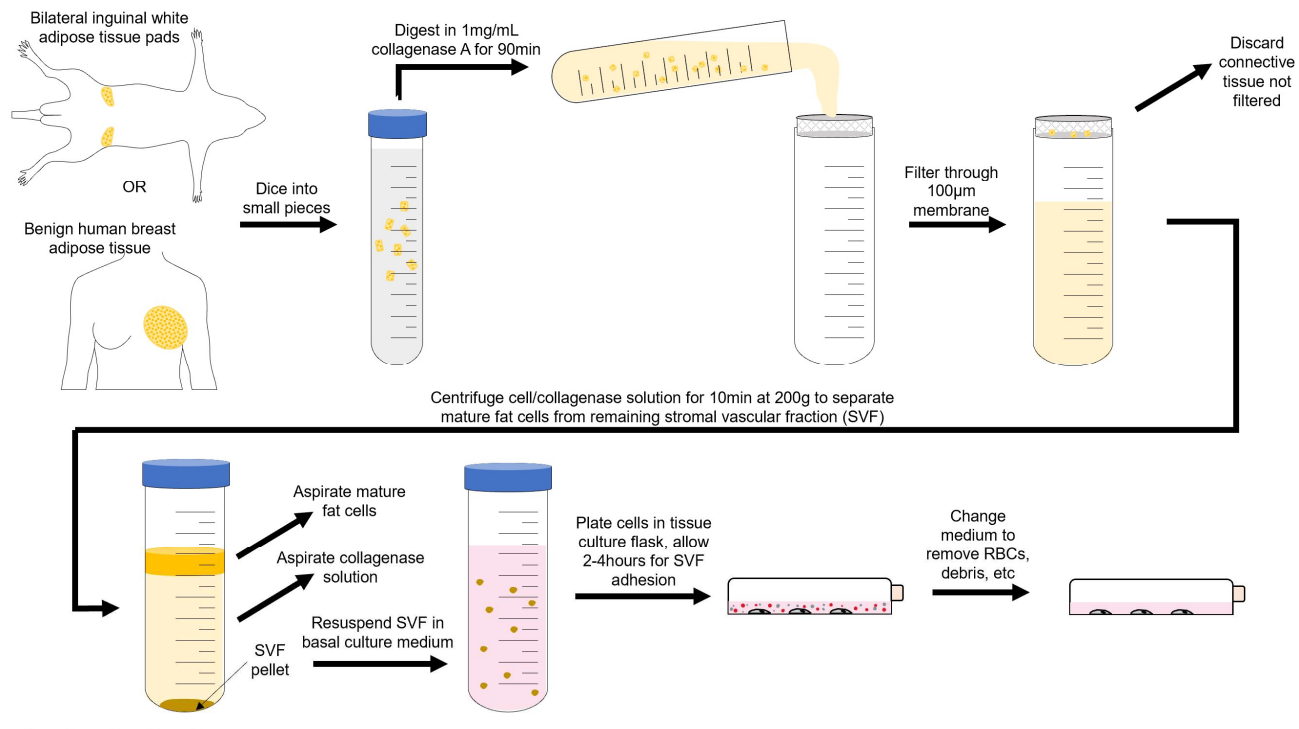


Figure 6. Process of SVF isolation for mouse and human adipose tissue samples

2.5 Immortalization of murine stromal vascular fraction cells

SVF cells were isolated from the inguinal fat pad of a 1-month old male C57BL/6J mouse as described above in section 2.4.1. SVF cells were immortalized with SV40 Large T antigen (Addgene plasmid #1780) as published previously [160]. Immortalized SVF cells were cultured in basal medium consisting of DMEM/F12 GlutaMAX supplemented with 200 U/mL penicillin/streptomycin and 10% FBS. These immortalized SVF cells are also referred to as mouse immortalized white preadipocytes, 'mIWPA,' throughout the text.

2.6 Differentiation of mature adipocytes

2.6.1 Mouse immortalized white preadipocytes (mIWPA) differentiation

mIWPA cells were grown in basal cell culture medium as described above in section 2.5. Cells were plated on cell culture plates coated with 0.1% gelatin solution (Sigma-Aldrich G1393) according to manufacturer protocol and grown to confluency in basal cell culture medium before differentiation. For white adipocyte differentiation, an induction cocktail comprised of basal medium, supplemented with 5µg/mL insulin (Sigma-Aldrich I9278), 1µM dexamethasone (Sigma-Aldrich D1756), 0.5mM 3-isobutyl-1-methylxanthine (IBMX, Sigma-Aldrich I5879), and 125µM indomethacin (Sigma-Aldrich I7378) was used for 2 days. After induction, differentiating white adipocytes were cultured in a maintenance cocktail comprised of basal medium supplemented with 5µg/mL insulin for 12 days. For beige adipocyte differentiation, induction cocktail comprised of basal medium supplemented with 5µg/mL insulin, 5µM dexamethasone, 0.5mM IBMX, 125µM indomethacin, 0.5µM rosiglitazone (Sigma-Aldrich R2408), and

1nM (T3, Sigma-Aldrich T6397) and used for 2 days. After induction, differentiating beige adipocytes were cultured in a maintenance cocktail comprised of basal medium supplemented with 5µg/mL insulin, 1µM rosiglitazone, and 1nM T3 for 12 days. See next subsections for experimental conditions after maturation. Medium for all cells was changed every 2-3 days. The maximum passage number used for any mIWPA experiments was passage 13.

2.6.2 Murine primary SVF differentiation

Primary mouse SVF cells were grown in a basal cell culture medium as described in section 2.4.1. Cells were plated on cell culture plates coated with 0.1% gelatin solution and grown to confluency in basal cell culture medium before differentiation. For white adipocyte differentiation, an induction cocktail comprised of basal medium without Normocin, supplemented with 5µg/mL insulin, 1µM dexamethasone, 0.5mM IBMX, and 125µM indomethacin was used for 4 days. After induction, differentiating white adipocytes were cultured in a maintenance cocktail comprised of basal medium without Normocin supplemented with 5µg/mL insulin for 12 days. For beige adipocyte differentiation, induction cocktail comprised of basal medium without Normocin, supplemented with 5µg/mL insulin, 5µM dexamethasone, 0.5mM IBMX, 125µM indomethacin, 0.5µM rosiglitazone, and 1nM T3 and used for 4 days. After induction, differentiating beige adipocytes were cultured in a maintenance cocktail comprised of basal medium without Normocin supplemented with 5µg/mL insulin, 1µM rosiglitazone, and 1nM T3 for 12 days. See next subsections for experimental

conditions after maturation. Medium for all cells was changed every 2-3 days. The maximum passage number used for any primary SVF experiments was passage 4.

2.6.3 Human primary SVF differentiation

Primary human SVF preadipocytes were grown in a basal cell culture medium as described in section 2.4.2. Cells were plated on cell culture plates coated with 0.1% gelatin solution and grown to confluency in basal cell culture medium before differentiation. For white adipocyte differentiation, an induction cocktail comprised of basal medium (either of the two basal mediums described in section 2.4.2) supplemented with 5µg/mL insulin, 1µM dexamethasone, 0.5mM IBMX, and 125µM indomethacin was used for 4 days. After induction, differentiating white adipocytes were cultured in a maintenance cocktail comprised of basal medium supplemented with 5µg/mL insulin for 12 days. For beige adipocyte differentiation, induction cocktail comprised of basal medium supplemented with 5µg/mL insulin, 5µM dexamethasone, 0.5mM IBMX, 125µM indomethacin, 0.5µM rosiglitazone, and 1nM T3 and used for 4 days. After induction, differentiating beige adipocytes were cultured in a maintenance cocktail comprised of basal medium supplemented with 5µg/mL insulin, 1µM rosiglitazone, and 1nM T3 for 12 days. See next subsections for experimental conditions after maturation. Medium for all cells was changed every 2-3 days. The maximum passage number used for any primary human SVF experiments was passage 6.

2.7 Cancer cells lines and culture

2.7.1 E0771

Murine E0771 cells (CH3 BioSystems 940001) were grown in basal cell culture medium consisting of RPMI1640+GlutaMAX (Thermo Fisher 61870-036), supplemented with 10% FBS and 200U/mL penicillin, 0.2mg/mL streptomycin, and 25µg/mL amphotericin B. This murine breast cancer cell line has a C57BL/6 background and is ER+. The maximum passage number used for any experiments was passage 5.

2.7.2 4T1

Two populations of murine 4T1 cells were used in experiments. One population was obtained at passage 3 from the laboratory of Dr. Harry Bear, and the other was purchased from ATCC (ATCC CRL-2539). Both were grown in basal cell culture medium consisting of RPMI1640+GlutaMAX supplemented with 10% FBS and 200 U/mL penicillin/streptomycin. This murine breast cancer cell line has a BALB/c background and has a more metastatic phenotype than E0771 cells. The maximum passage number used for any experiments was passage 7.

2.7.3 LLC

Murine Lewis lung carcinoma (LLC) cells were obtained at passage 8 from the laboratory of Dr. David Gewirtz, and grown in basal cell culture medium consisting of DMEM/F12+Glutamax supplemented with 10% FBS and 200 U/mL penicillin/streptomycin. The maximum passage number used for any experiments was passage 11.

2.7.4 MCF-7

Human MCF-7 cells were obtained at passage 10 from the laboratory of Dr. Kazuaki Takabe and grown in basal cell culture medium consisting of RPMI1640+GlutaMAX supplemented with 10% FBS and 200 U/mL penicillin/streptomycin. This cell line of human breast adenocarcinoma is 'luminal' type A (ER+, PR+, HER2-). The maximum passage number used for any experiments was passage 13.

2.7.5 MDA-MB-231

Human MDA-MB-231 cells were obtained at passage 12 from the laboratory of Dr. Kazuaki Takabe, and grown in basal cell culture medium consisting of RPMI1640+GlutaMAX supplemented with 10% FBS and 200 U/mL penicillin/streptomycin. This cell line of human breast adenocarcinoma is 'basal' type and triple negative (ER-, PR-, and HER2-). The maximum passage number used for any experiments was passage 15.

2.8 Cancer conditioned medium and coculture experiments

For conditioned medium collection, cells were grown to ~70% confluency, and then cell culture medium was changed. This conditioned medium was harvested 24 hours later and filtered through a 0.22µm pore size filter (Millipore SLGP033RS) for use in experiments. For coculture experiments, cells were grown to ~70% confluency on coculture inserts (Fisher Scientific 08-771-21) and then moved to the appropriate adipocyte wells for experiments. Empty coculture wells were placed in all other

adipocyte wells. The maximum passage number used for any experiments was passage 11.

2.8.1 mIWPA experiments

mIWPA cells were grown and induced to either mature white or beige adipocytes as described in section 2.6.1. On day 14, mature white preadipocytes were exposed to white maintenance medium plus one of the following for 24 hours: 1) no treatment (white control) or 2) cancer conditioned medium (E0771 or LLC) in a 1:1 ratio with white maintenance medium cocktail. Mature beige adipocytes continued on beige maintenance medium (beige control). Schematic of exposure periods visualized in Figure 7.

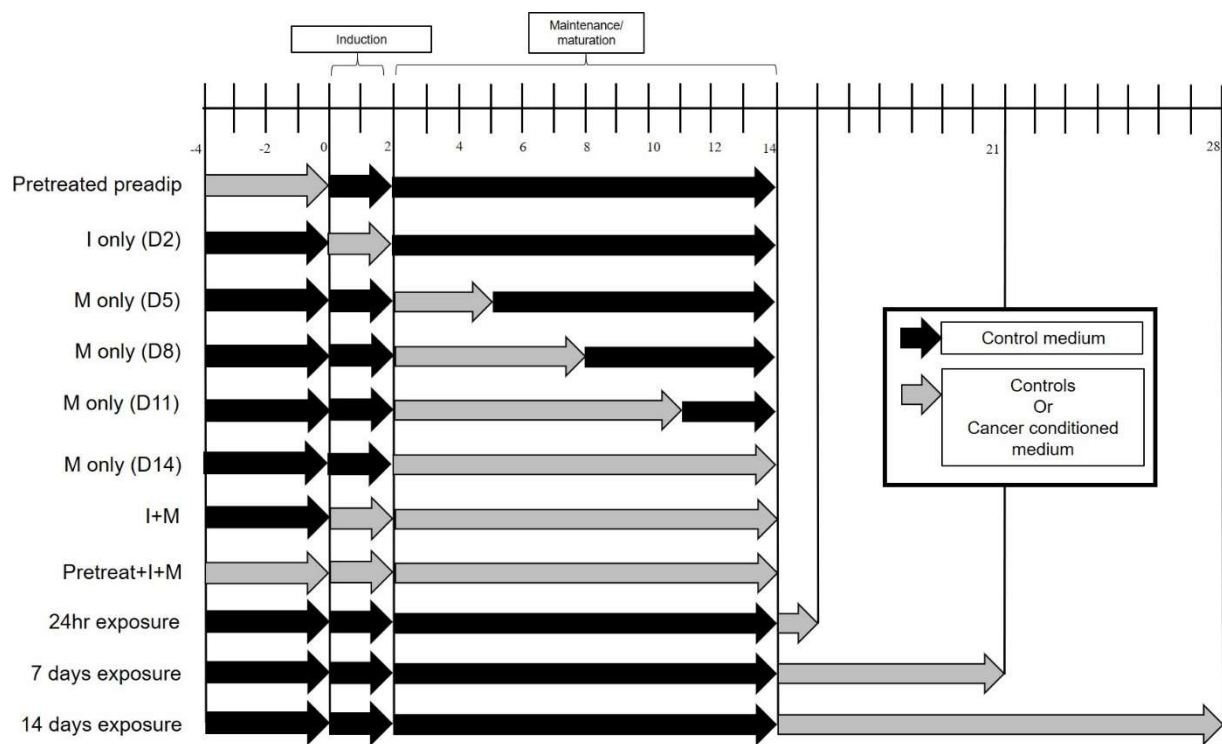


Figure 7. Murine immortalized SVF experiment schematic

2.8.2 Primary murine SVF experiments

Primary murine SVF cells were isolated, grown, and induced to either mature white or beige adipocytes as described in section 2.6.2. On day 16, mature white preadipocytes were exposed to white maintenance medium plus one of the following for 24 hours: 1) no treatment (white control), 2) 10 μ M CL 316,243 hydrate (Sigma-Aldrich S5976), 3) cancer conditioned medium (either E0771 or 4T1) in a 1:1 ratio with white maintenance medium cocktail, or 4) coculture (with either E0771 or 4T1). Mature beige adipocytes continued on beige maintenance medium (beige control). Schematic of exposure periods visualized in Figure 8.

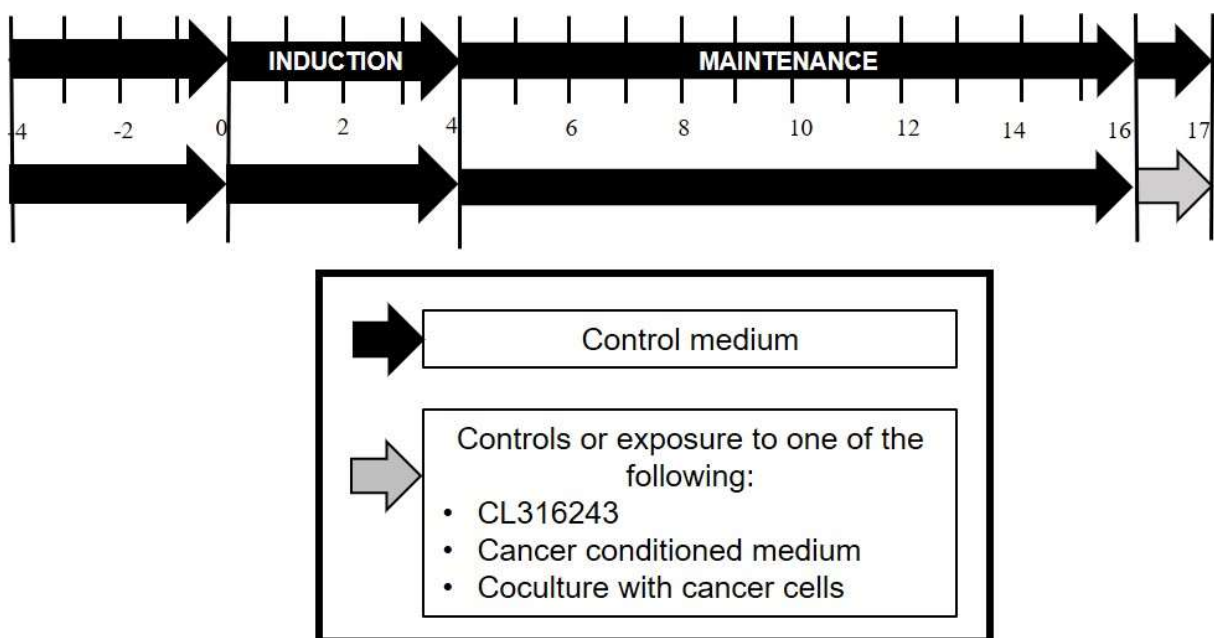


Figure 8. Murine primary SVF experiment schematic

2.8.3 Primary human SVF experiments

Primary human SVF cells were isolated, grown, and induced to either mature white or beige adipocytes as described in section 2.6.3. On day 16, mature white preadipocytes were exposed to white maintenance medium plus one of the following for 24 hours: 1) no treatment (white control) or 2) cancer conditioned medium (either MCF-7 or MDA-MB-231) in a 1:1 ratio with white maintenance medium cocktail. Mature beige adipocytes continued on beige maintenance medium (beige control). Schematic of exposure periods visualized in Figure 9.

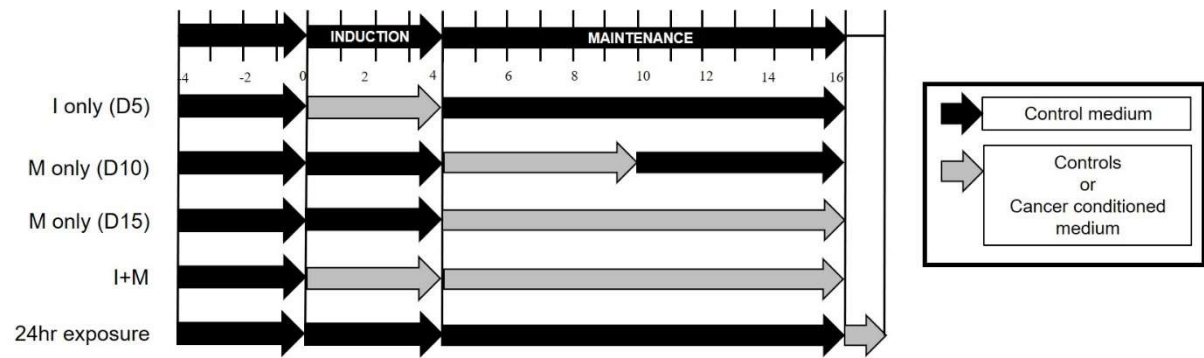


Figure 9. Human primary SVF experiment schematic

2.9 IL6 experiments

Mature adipocytes from primary murine SVF cells were prepared as described in section 2.6.1. On day 16, mature white preadipocytes were exposed to white maintenance medium plus one of the following for 3 days: 1) no treatment (white control), 2) 10 μ M CL 316,243, 3) 40ng/mL mouse recombinant IL6 (R&D Systems 406-ML/CF), 4) 40ng/mL mouse recombinant IL6 + 200ng/mL mouse recombinant IL6RA (R&D Systems, 1830-SR/CF). Mature beige adipocytes continued on beige maintenance medium (beige control). Schematic of experiment visualized in Figure 10.

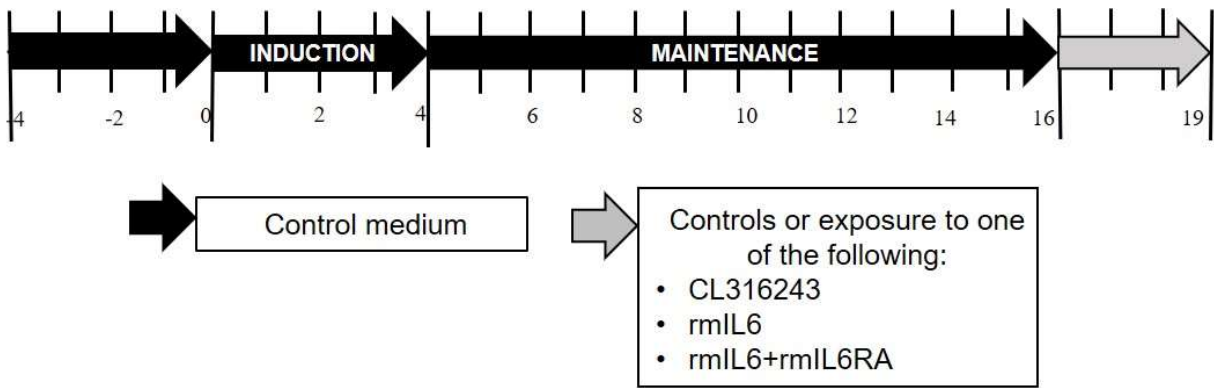


Figure 10. IL6 experiment schematic

2.10 Tumor lysate & CD11b+ cell experiment

In collaboration with the laboratory of Dr. Paula Bos, tumor lysate and isolated CD11b+ cells were obtained from the following murine models of breast cancer: MMTV-PyMT at an early stage (10 weeks old at time of harvest), MMTV-PyMT at a late stage (24 weeks old at time of harvest), PyMT-derived cell line injected into bilateral inguinal mammary pads of a C57BL/6J mouse (6-8 weeks old age at time of injection, 150,000 cells injected, harvested when tumors reach 2000mm³), E0771 cell line injected into bilateral inguinal mammary pads of a C57BL/6J mouse (6-8 weeks old age at time of injection, 500,000 cells injected, harvested 10 days later). All mice were female.

For tumor lysate isolation, mice were euthanized via cervical dislocation. Tumors were dissected from mice and immediately frozen on dry ice for 10 minutes. Tissue was powdered using a mortar and pestle on dry ice. Cytokine lysis buffer consisting of (for every 50mL total): 2.5mL of 50mM Tris pH 7.5 (Fisher), 7.5mL of 150mM NaCl (Fisher), 2.5mL of 1% NonidetP-40 (Fisher), 0.1mL of 1mM EDTA (Fisher), 37.4mL of H₂O, and 1x protease inhibitor cocktail (SIGMA cat#4693132001) was made. Frozen tissue powder was incubated in cytokine lysis buffer at a ratio of 1-2μL buffer for every 1mg of tissue for one hour in a cold room on a rotator. Solution was then sonicated three times at an amplitude of 40μm, 10 seconds each time with 30 seconds rest on ice in between. The tissue homogenate was then centrifuged three times at 14000rpm at 4°C for 10 minutes each. In between each centrifugation, supernatant was collected and moved to a new tube for the next centrifugation, and pellet (cell debris, etc) was discarded. After final centrifugation and transfer of supernatant (tumor lysate) to final tube, lysate protein concentration was quantified using the Thermo Pierce BSA Protein Assay Kit (Thermo

23225) using the microplate protocol. Various dilutions of lysate were used to ensure that sample would be within the standard's working range for quantification (examples include 1:1, 1:10, 1:50 in cytokine lysis buffer). Absorbance was measured at 560nm using a POLARstar OPTIMA plate reader. Program software was used to calculate protein concentrations. Tumor lysate was frozen at -80°C until needed.

Isolation of CD11b+ cells was performed using Dynabeads magnetic separation technology. Mice were euthanized via cervical dislocation. Tumors near inguinal fat pads were removed from mice, careful to exclude lymph nodes, rinsed in ice-cold PBS, and then minced in 1x LiberaseTL (Roche). Homogenate was incubated for 30 minutes on a rotator in a 37°C incubator. 10mL of cell medium consisting of DMEM (Gibco), 10%FBS (WZ), and penicillin/streptomycin (Gibco) was added to homogenate, and then filtered using 100µm pore size filters into 50mL tubes, then flushed with an extra 10mL of cell culture medium. Everything from this point was performed under sterile conditions in the lab's cell culture room. Cells were centrifuged at 350g for 5 minutes at 4°C. Supernatant was removed and cells were resuspended in 8mL of 43% Percoll (GE) + 2% FBS (VWR). Cells were centrifuged at 500g for 8 minutes at 4°C. Supernatant was removed again and cells were washed with 2mL of MACS sorting buffer (0.5% BSA, 2mM EDTA) before another centrifugation at 350g for 5 minutes at 4°C. Supernatant was removed and cells were resuspended in 300µL of MACs sorting buffer and incubated in 200µL of Fc block (2.4g2) and 4µL of biotinylated CD11b monoclonal antibody (eBioscience 13-0112-86) for 30 minutes in cold room on a rotator. Magnetic bead master mix was created by diluting 100µL of Dynabeads (Biotin Binder, Invitrogen 11047) in 2mL of MACs in a 15mL conical tube (multiples were created when

needed). Tubes were placed in a magnet (DynaMag™-15 Invitrogen 12301D) and remaining liquid was removed, leaving only washed magnetic beads. These beads were then resuspended in 100µL of MACs. 100µL of Magnetic bead master mix and 200µL of MACs was added to each tube of cells after incubation in Fc blocker and antibody. Tubes were placed in magnet for about 2 minutes, and liquid supernatant was discarded (CD11b+ cells should be attached to beads which went to magnet). Remaining cells conjugated to beads were resuspended in 500µL of MACs and 500µL of release buffer (Dynabead component-same catalog number) and incubated for 10-20 minutes on a rotator in the cold room. After this, 1mL of MACs was added to each tube and tubes were placed on magnet. Liquid supernatant was collected into a new tube and beads were discarded. Suspended cells were centrifuged at 350g for 5 minutes at 4°C, supernatant removed, and then cells were resuspended in 1mL of cell culture medium. Cells were counted using an automated cell counter (Life Technology Countess II) with Trypan blue (1:1 with cells) for live-dead differentiation. Live cell count was recorded, and cells were kept on ice until plated.

Primary C57BL/6J SVF cell lines were isolated as described in section 2.6.2. Cells were plated on to gelatin-coated 12 well plates at passage 4 at a density of 3000 cells/cm². Basal cell culture medium was changed every other day until cells reached full confluency, at which point induction and maintenance mediums were used (compositions described in section 2.6.2). After 4 days of induction followed by 15 days of maintenance medium, some W and B wells were harvested for baseline mRNA, protein, and lipid droplet visualization analyses (see sections 2.14, 2.15, and 2.16, respectively). For the remaining wells, medium was changed to either continued white

or beige maintenance medium for controls, or to continue white maintenance medium plus one of the following experimental conditions (concentrations/amounts are for each well exposed): 40ng/mL of mouse recombinant IL6; 40ng/mL recombinant mouse TNF α (R&D Systems 410-MT-010/CF); 10uM CL 316,243 hydrate; 4 μ g of either MMTV early tumor lysate, MMTV late tumor lysate, Pymt tumor lysate, E0771 tumor lysate; 0.6 μ L of cytokine lysis buffer without protease inhibitor cocktail as a control for lysates; 28,000 Pymt CD11b $^{+}$ cells; 15,000 E0771 CD11b $^{+}$ cells (pan myeloid); 1mL of E0771 cancer conditioned medium (see cancer conditioned medium experiments); 1mL RPMI-based culture medium as a control for cancer conditioned medium. Cells were harvested after 3 days of exposure (Figure 11).

2.11 4T1 allograft experiment

In collaboration with the laboratory of Dr. Daniel Conrad, nine BALB/c wild-type (WT) (4 female, 5 male) and nine BALB/c *Rag1*^{-/-} mice (4 female, 5 male) were used for this 4T1 allograft experiment.

4T1 cells were prepared for injection via the following protocol: cells were cultured in their basal culture medium in 150mm flasks until confluent. Once cells reached confluency, flasks were washed with 1X PBS. Cells were scraped and centrifuged at 1500rpm for 5 minutes at 21°C. Supernatant was removed and cells were resuspended in 5mL 1X PBS and kept on ice to prevent clumping. 10µL of cell suspension was mixed with 10µL of 2% trypan blue to count live cells. 1X PBS was added to achieve concentration of 1x10⁶ cells/mL.

Mice were inoculated with either 200,000 4T1 cells in 200µL PBS ('4T1 group') or 200µL PBS (vehicle control, 'control group') unilaterally in the right inguinal area using a 26 gauge needle at the following ages for the mice (

Figure 12): WT females 62 days (about 9 weeks); WT males 49 days (7 weeks); *Rag1*^{-/-} females 76 days (about 11 weeks); *Rag1*^{-/-} males 57 days (about 8 weeks). Mice were kept in cages (separated based on sex and genotype) at 23°C. Mice were weighed at baseline and every other day. Injection site palpated every other day. Once tumor became palpable, tumor size was measured with calipers every day.

4T1 group mice were euthanized 10 days after inoculation. Control group mice were euthanized 14 days after inoculation. Rectal temperature was obtained using a rectal temperature probe for all mice prior to euthanization. Mice were euthanized in isoflurane. Tumor (if present) and adipose tissues (interscapular, axillary, inguinal, and

gonadal) were harvested for gene expression and flow cytometry analyses (see sections 2.14 and 2.12, respectively).

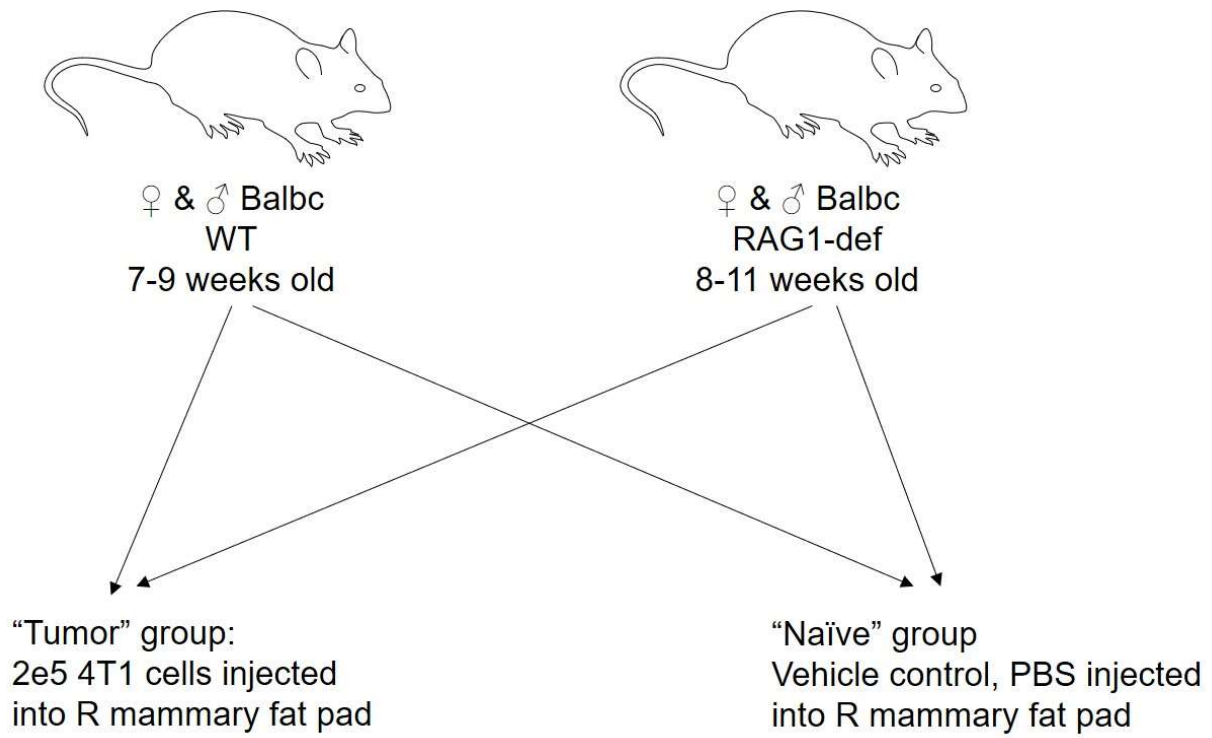


Figure 12. 4T1 allograft experiment schematic

2.12 Flow cytometry

Data for both cell-surface and intracellular flow cytometry were acquired on the LSR Fortessa flow cytometer (BD Biosciences) in the VCU Flow Cytometry Core. Data were analyzed using FCS Express 5 (De Novo Software).

2.12.1 Cell surface flow cytometry

Cell surface flow cytometry staining was performed as described previously [161] for either whole tissue or cells grown in cell culture:

For cells grown *in vitro*, primary murine SVF were isolated, grown, and induced to either mature white or beige adipocytes as described in section 2.6.1 until day 16. Cells were gently washed twice with 1X PBS. Staining solution was added to each well (for each well on 12-well plate: 250µL total volume consisting of respective targets (Table 2) with Fc blocker diluted in 1X PBS) and left to incubate at 4°C in the dark for 20 minutes. 100µL of live-dead stain (Biolegend Zombie Aqua Dye 77143 in DMSO) diluted 1:100 in 1X PBS was added to each well (still had staining solution it) and incubated for 15 minutes at room temperature in the dark. 350µL of 1X FACS with Azide was added to each well for 5 minutes at room temperature. Cells were then gently scraped off of the well and moved to a FACS tube. Suspension was pipetted up and down several times to encourage single-cell suspension. 700µL of Fixation buffer (Biolegend 420801) was added to each sample and left to incubate for 20 minutes at room temperature. Tubes were then centrifuged at 250g for 10 minutes at room temperature. Sheets and clumps of cells remained floating at the top, some cellular material floated in the middle, and a small amount of cellular material precipitated to the bottom. The top and middle

portions were discarded, and the bottom portion was resuspended in 300 μ L of FACS with Azide, pipetting up and down several times to encourage single-cell suspension. Samples were passed through a 100 μ m mesh filter before running on flow cytometry instrument.

For whole tissue, tissue samples were placed in 12 well dishes with 1mL of 1mg/mL collagenase A and homogenized. For each sample, homogenized tissue and collagenase was moved to a 2mL Eppendorf tube and placed on a rotator in a 37°C incubator for about 45 minutes. Homogenized solution was filtered into 50mL tube with a 70 μ m filter (Fisherbrand 22363548). Filtered cell solution was centrifuged at 1500rpm for 5 minutes at room temperature. Supernatant was decanted and 100 μ L of 1X mojosort was added. This mixture was then moved to a well on a 96 well plate and kept on ice. Once all samples were loaded, the plate was centrifuged at 2000rpm for 4 minutes at room temperature, decanted, and washed with 200 μ L of 1X PBS per well. This was repeated twice. 100 μ L of live-dead stain (Biolegend Zombie Aqua Dye 77143 lot B194251 in DMSO) diluted 1:100 in 1X PBS was added to each well and incubated for 10 minutes at room temperature in the dark. Plate underwent two cycles of 100 μ L 1X mojosort buffer wash per well, centrifuged, and decanted. 50 μ L of 2.4g2 [162] to block Fc receptors (diluted in 1X mojosort) was added to each well and incubated for 10 minutes at room temperature. 50 μ L of antibody mix per well (25 μ L brilliant blue stain buffer, 0.5 μ L of each antibody used (Table 2), and the remaining with 1X mojosort) was added to each well and moved to refrigerator to incubate for 30 minutes. Plate was centrifuged, decanted, and then samples washed with 200 μ L 1X mojosort per well before another cycle of centrifugation and decanting. Cells were then fixed with 150 μ L

of 1X fixing solution for 10 minutes at room temperature. Plate was centrifuged and decanted. 200µL of 1X mojosort was added to each well and the solution in each well was transferred to individual tubes for running flow cytometry.

2.12.2 Intracellular flow cytometry

For intracellular cytokine staining, cells (mIWPA, E0771, LLC, 4T1, mouse peritoneal cavity cells) were cultured in 6 or 12 well plates for each cell type until ~70% confluent. Murine peritoneal cavity cells were isolated by lavage the day preceding each experiment by the Conrad laboratory. Cells were treated with one of the following conditions in basal medium before harvest: 1) 1X Brefeldin A (BioLegend 420601) and 1X Monensin (BioLegend 420701) for 6 hours; 2) 1X Brefeldin A, 1X Monensin, 1X phorbol 12-myristate-13-acetate (PMA; Sigma-Aldrich P1585) and 1X Ionomycin (ThermoFisher I24222) for 3 hours; 3) no treatment.

Wells of same treatment group were pooled together and harvested with trypsin, pelleted, and resuspended in 200µL of 1X mojosort to encourage single cell suspensions. 100µL of cell suspension moved into two wells of a 96-well plate on ice. The 96-well plate was contributed at 2000rpm for 3 minutes at room temperature, decanted, washed with 200µL of 1X PBS per well, spun again, and decanted. 100µL of live-dead stain was added per well (Biolegend Zombie Aqua in DMSO, diluted 1:200 in 1X PBS) and left to incubate in the dark at room temperature for 10 minutes. Wells were washed with 100µL 1X FACS with Azide, spun at 2000rpm for 4 minutes at room temperature, decanted, and washed again with 180µL 1X mojosort, spun, decanted. 100µL of 2.4g2 was added in 1X FACS (40µL 2.4g2 in 4mL FACS)) for 5 minutes at

room temperature, then spun and decanted. 150µL of fixation buffer was added per well and left to incubate in the dark at room temperature for 15 minutes. Plate was spun, decanted. Wells were washed, spun, decanted with 170µL of 1X permeabilization buffer (Biolegend 421002 diluted to 1X in H₂O) twice. Intracellular stain mixture was made to get 100µL per well: 98µL of 1X perm buffer, 1µL antibody (Table 2), and 1µL of Fc blocker. 100µL of intracellular stain mixture was added and incubated for 40 minutes in the dark at room temperature. Plate was then spun, decanted. Wells were washed twice with permeabilization buffer, 170µL per well each time, spun, and decanted. Wells were then washed with 170µL 1X FACS to seal the membrane, spun, decanted. 150µL of fixation buffer was added to each well and incubated at room temperature in the dark for 15 minutes. Plate was spun and decanted. Wells were resuspended in 200µL FACS and transferred to flow tubes. An additional 100µL FACS was added to each tube to get 300µL per tube. Tubes were covered in foil and placed in the refrigerator for storage before flow cytometry was performed.

Table 2. Flow cytometry antibodies

Target	Isotype	Stain/ Dye	Clone	Catalog Number	Lot Number	Company	[] Used	Use
CD45	Rat IgG2b, κ	APC/Fire 750	30-F11	103154	-	Biolegend	2 μ g/mL	Cell surface
Ly6G	Rat IgG2a, κ	Brilliant Violet	D7	108131	-	Biolegend	2 μ g/mL	Cell surface
Fc ϵ R	Rat IgG2a, κ	FITC	1A8	127606	-	Biolegend	2 μ g/mL	Cell surface
CD3 ϵ	Hamster IgG	PE/Cy7	145-2C11	100320	-	Biolegend	2 μ g/mL	Cell surface
CD45R/B 220	Rat IgG2a, κ	PE/Cy7	RA3-6B2	103222	-	Biolegend	2 μ g/mL	Cell surface
I-A/I-E	Rat IgG2b, κ	Brilliant Violet	M5/114	107632	-	Biolegend	2 μ g/mL	Cell surface
CD11c	Hamster IgG	Brilliant Violet 605	N418	117334	-	Biolegend	2 μ g/mL	Cell surface
Ly6C	Rat IgG2c, κ	APC	HK1.4	17-5932-80	-	eBiosciences	2 μ g/mL	Cell surface
F4/80	Rat IgG2a, κ	PE	BM8	123110	-	Biolegend	2 μ g/mL	Cell surface
CD11b	Rat IgG2b, κ	BUV395	M1/70	563553	-	BD Horizon	2 μ g/mL	Cell surface
Fc ϵ R1 α	Hamster IgG	PE/Dazzl e	MAR-1	134332	-	Biolegend	2 μ g/mL	Cell surface
Mouse IL- 6	Rat IgG1, κ	PE	MP5-20F3	554401	7159590	BD Biosciences	2 μ g/mL	Intracellular
-	Rat IgG1, κ	PE	R3-34	554685	7026874	BD Biosciences	2 μ g/mL	Intracellular isotype control
Mouse GP130 (CD130)	Rat IgG2b, κ	PE	4H1B35	149404	B248938	Biolegend	2 μ g/mL	Cell surface
-	Rat IgG2b, κ	PE	RTK4530	400607	B255376	Biolegend	2 μ g/mL	Cell surface isotype control
Mouse IL6RA (CD126)	Rat IgG2b, κ	APC	D7715A7	115812	B249718	Biolegend	2 μ g/mL	Cell surface
-	Rat IgG2b, κ	APC	RTK4530	400611	B261320	Biolegend	2 μ g/mL	Cell surface isotype control

2.13 RNA isolation and cDNA generation

Total RNA was isolated with TRIzol (Thermo Fisher 15596018) according to manufacturer protocol for whole tissue or culture cells. Isolation was performed with UltraPure Glycogen (Thermo Fisher 10814-010). RNA was quantified using the NanoDrop 1000 Spectrophotometer (Thermo Fisher). cDNA was generated using the BioRad iScript cDNA Synthesis Kit (BioRad 170-8891) with an input of 1µg of RNA on a C1000 Thermal Cycler (BioRad). The cDNA was then diluted 1:3 in molecular grade water.

2.14 Quantitative PCR (qPCR)

qPCR was performed on the QuantStudio 3 Real-Time PCR System (Thermo Fisher) with 10µL per reaction on 96 well plates in technical duplicates or triplicates: 1X PowerUp SYBR Green Master Mix (Applied Biosystems A25778), forward and reverse primers (0.5µM for mouse, 1µM for human; refer to Table 3 and Table 4 respectively for sequences and information), and 3µL of diluted cDNA. The Thermo Fisher Cloud platform was used for analysis of efficiency-corrected C_q values using endogenous control (*Tbp* for mouse, *TBP* for human), and fold-changes ($2^{\Delta\Delta C_q}$) were calculated using Microsoft Excel.

Table 3. Mouse primers used for qPCR

Gene	Efficiency	Full Name	NCBI Gene ID	NCBI Reference Sequence	Product Length (bp)	Variants Targeted	Forward Primer (5'-3')	Reverse Primer (5'-3')
<i>Cfd</i>	97.247	complement factor D (adipsin)	11537	NM_013459.3	100	V1, V2, V3	AACCGGACA ACCTGCAATCT	AGTCTCCCCT GCAAGTGTCC
<i>Cidea</i>	99.083	cell death-inducing DNA fragmentation factor, alpha subunit-like effector A	12683	NM_007702.2	197	<i>Cidea</i> mRNA	TCCTCGGCT GTCTCAATG	TGGCTGCTCT TCTGTATCG
<i>Dll1</i>	97.176	Delta like non-canonical Notch ligand 1	13386 (aka Pref-1)	NM_010052.5	109	V1-V5; X1-X2	AAGTGTGTA ACTGCCCCTGG	TGCAAGCCCG AACGTCTATT
<i>Fasn</i>	99.309	fatty acid synthase	14104	NM_007988.3	104	<i>Fasn</i> mRNA	TGCACCTCA CAGGCATCAAT	GTCCCACTTG ATGTGAGGGG
<i>Il6</i>	93.237	Interleukin-6	16193	NM_031116.2	79	V1, V2	CCACTTCAC AAGTCGGAGGC	TTGCCATTGC ACAACTCTTTCT
<i>Il6ra</i>	97.549	interleukin 6 receptor, alpha	16194	NM_010559.3	104	V1, V2	TGAATGATG ACCCCAAGGCAC	ACACCCATCC GCTCTCTACT
<i>Il6st</i>	99.644	interleukin 6 signal transducer aka Gp130	16195	NM_010560.3	60	<i>Il6st</i> mRNA	TTACAACAC CAAAGTTCGCTCA	CTAAGCACAC AGGCACGACTA
<i>Lep</i>	91.876	leptin	16846	NM_008493.3	131 (128 variant X1)	<i>Lep</i> mRNA, X1	GCACTGCCT ATCCAGAAA GTCC	GGAATGAAGT CCAAGCCAGTGAC
<i>Lipe</i>	100.441	lipase, hormone sensitive	16890	NM_010719.5	150	V1,V2 X1,X2,X3	CGCTACACA AAGGCTGCTCTC	AACTGGCGGT CACACTGAG
<i>Lpl</i>	90.259	lipoprotein lipase	16956	NM_008509.2	113	<i>Lpl</i> mRNA	GCGTAGCAG GAAGTCTGACCA	AGCGTCATCA GGAGAAAGGCGA
<i>Mgll</i>	108.576	monoglyceride lipase	23945	NM_0011166251.1	72	V1, V4	CGCGAGGTT TCCTTCCCTAA	AAAGTCACCC CGATTCTCCG
<i>Oplah</i>	98.557	5-oxoprolinase	75475	NM_153122.2	108	Predicted isoforms	CTGCTGGTG GGTTCTCCC	CCACGGTCGA TGGCAAATG
<i>Pparg</i>	98.04	peroxisome proliferative activated receptor, gamma	19016	NM_0111146.3	73	V2, X8	GCATGGTGC CTTCGCTGA	TGGCATCTCT GTGTCAACCATG
<i>Pparg c1a</i>	99.095	peroxisome proliferative activated receptor,	19017	NM_008904.2	52	V1 mRNA, V2 ncRNA	ATGTGCAGC CAAGACTCTGT	CAACCAGAGC AGCACACTCT

		gamma, coactivator 1 alpha						
<i>Prdm16</i>	97.145	PR domain containing 16	70673	NM_027504.3	87	V1-4, X1-7	CAGCACGGTGAAGCCATT C	GCGTGCATCCGCTTGTG
<i>Ptk2b</i>	87.917	Protein-tyrosine kinase 2-beta	19229	NM_001162366.1	81	V1-V3, X1-X7	TGCAGACAGAGATCCAGG AGA	TATTCGCCCACTCAGGAGGA
<i>Slc27a1</i>	99.235	long-chain fatty acid transport protein 1	26457	NM_011977.3	106	Plus other predicted	GGGATCTCTCTCTCCACC CC	GAGCTTGCCTGATCCAGCTT
<i>Slc29a1</i>	96.822	equilibrative nucleoside transporter 1	63959	NM_001199113.1	120	V1-V5; other predicted	CACCAGCCTCAGGACAGG TAT	GTCCAGGCGGTTTGTGAAA
<i>Socs3</i>	110.721	Suppressor of cytokine signaling 3	12702	NM_007707.3	122	mRNA, predicted variant X1	TAGACTTCA CGGCTGCCAAC	CGGGGAGCTAGTCCCGAA
<i>Tbp</i>	94.570	TATA box binding protein	21374	NM_013684.3	98	<i>Tbp</i> mRNA	AGAACAACAGCCTTCCAC CT	GCCGTAAGGCATCATTGGAC
<i>Tnfrsf9</i>	97.105	tumor necrosis factor receptor superfamily, member 9 (aka <i>Cd137</i>)	21942	NM_011612.2	104	V1,V2,V3, X1	CGTGCAGAACTCCTGTGA TAAC	GTCCACCTATGCTGGAGAAG G
<i>Ucp1</i>	95.837	uncoupling protein 1 (mitochondrial, proton carrier)	22227	NM_009463.3	91	<i>Ucp1</i> mRNA	CACCTTCCC GCTGGACAC T	CCCTAGGACACCTTTATACC TAATGG

Table 4. Human primers used for qPCR

Gene	Efficiency	Full Name	NCBI Gene ID	NCBI Reference Sequence	Product Length (bp)	Variants Targeted	Forward Primer (5'-3')	Reverse Primer (5'-3')
<i>EAR2</i>	105.984	Nuclear receptor subfamily 2 group F member 6	2063	NM_005234.3	81	<i>EAR2</i> mRNA	TACACCTGC CGGTCCAA	ACCCGGAAGC ACTTCTTGAG
<i>OPLAH</i>	103.185	5-oxoprolinase	26873	NM_017570.4	106	<i>OPLAH</i> mRNA	GAGCCGAGC GCCAGTTTT C	GTCGATGGCA AAGTGAAGC
<i>PDK4</i>	103.216	[Pyruvate dehydrogenase (acetyl-transferring)] kinase isozyme 4, mitochondrial	5166	NM_002612.3	65	<i>PDK4</i> mRNA	CCAAGCCAC ATTGGAAGC AT	TCAAAGGCAT CTTGGACCAC
<i>PPARGC1A</i>	93.971	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha	10891	NM_013261.3	91	<i>PPARGC1A</i> mRNA	TGACTGGCG TCATTCAGG AG	TGACTGGCGT CATTTCAGGAG
<i>PPARG</i>	94.305	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha	5468	NM_015869.4	70	<i>PPARG</i> mRNA, and predicted variants	ACCCAGAAA GCGATTCTT CA	GTGTCAACCA TGGTCATTT TTGT
<i>SLC27A1</i>	99.693	Long-chain fatty acid transport protein 1	376497	NM_198580.2	66	<i>SLC27A1</i> mRNA, and predicted variants	TGTACCACT CGGCAGGAA AC	GGACGACTGT CAGCCCATAG
<i>SLC29A1</i>	99.144	Equilibrative nucleoside transporter 1	2030	NM_001078177.1	100	<i>SLC29A1</i> mRNA, and predicted variants	ATCGATCTG GAGCCCGTG GA	ATCGATCTGG AGCCCGTGGA
<i>TBP</i>	102.419	TATA-box-binding protein	6908	NM_003194.4	68	<i>TBP</i> mRNA	CGGCTGTTT AACTTCGCTT CC	CAAGAAACAG TGATGCTGGG TC
<i>TMEM26</i>	104.328	Transmembrane protein 26	219623	NM_178505.6	111	<i>TMEM26</i> mRNA	TGACCTGGC AGTACAGAA CG	ACGCTGATTC CGATGTTCCA
<i>UCP1</i>	101.358	Uncoupling protein 1	7350	NM_021833.4	51	<i>UCP1</i> mRNA	GGCTTTCTT CAAGGGGTT GGTA	TTCCAGGATC CAAGTCGCAA

2.15 Protein quantification & western blotting

If protein samples were stored in -80°C freezer, they were thawed on ice prior to isolation and quantification. Protein isolation was performed using RIPA lysis and extraction buffer (Thermo Scientific 89901). RIPA buffer was supplemented with protease inhibitor (Roche 4693116001) and phosphatase inhibitors (Roche 4906837001) (1 tablet each per 10mL of RIPA buffer). 1µL of 1X nuclease solution (Pierce 88701, diluted in supplemented RIPA buffer) was added to each sample and placed on rotator at room temperature for 10 minutes. Samples were then centrifuged at 4°C at 13,000rcf for 10 minutes.

Protein quantification was performed using Pierce BCA Protein Assay Kit (Thermo Scientific 23227) according to manufacturer's 'microplate' protocol. Absorbance was measured at 562nm using a VersaMax microplate reader (Molecular Devices). And standard curve was generated using a four-parameter curve with SoftMax Pro 5.3 software. This standard curve was then used to determine the protein concentration of unknown samples.

Protein lysates were prepared for separation using 20µg of sample per well, with 1X NuPAGE LDS loading buffer (Invitrogen NP0007) and 1X NuPAGE reducing agent (Invitrogen NP0009). These samples were then 'boiled' on a 70°C heat block for 10 minutes. Samples were separated on NuPAGE 4-12% Bis-Tris Midi Gels (ThermoFisher WG1401BOX) using an XCell4 Surelock Midi-Cell (Invitrogen) and 1X NuPAGE MOPS SDS running buffer (Invitrogen NP0001, diluted in H₂O). 8µL of pre-stained protein ladder (Fisher 26616) was loaded in first and/or last well. Gel transfer was performed

using the iBlot Transfer Stack, PVDF, regular size (Invitrogen IB401001) 'dry blot transfer' method.

Membranes were washed with distilled H₂O and then 100% methanol. Membranes were left to air-dry and were re-wetted in 100% methanol before being transferred to box with 5% non-fat milk solution (BioRad 170-6404, diluted in 1X TBST). Membranes were left for blocking on a rocker at room temperature for 1 hour. Membranes were then washed once in TBST, cut as necessary for appropriate targets, and moved into separate tubes for primary antibody incubation (primary antibodies in Table 5 diluted in 1% BSA in 1X TBST) on a rotator in cold room overnight.

The next day, membranes were washed three times in 1X TBST on a shaker at room temperature for 10 minutes each. Membranes were moved to black boxes for secondary antibody incubation (secondary antibodies in Table 5 diluted in non-fat milk solution saved from prior day) on a rocker at room temperature for one hour. Membranes were then washed with 1X TBST three times on a shaker for 10 minutes each.

Membranes were incubated in chemiluminescence substrates for 1-2 minutes (Cell Signaling 7003, both diluted to 1X in distilled H₂O) to detect western blot signal. Images were captured either using film or the ChemiDoc MP Imaging System (BioRad).

Stat1/2/3/5 Control Cell Extracts (Cell Signaling 9133) were used for phosphorylated STAT3 negative and positive controls.

Table 5. Western blot antibodies

Target	Isotype	Stain / Dye	Clone	Catalog Number	Lot Number	Company	Concentration Used	Use
Phospho-STAT3 (Tyr705)	Rabbit IgG	-	D3A7	9145 S	34	Cell Signaling	1:2000	Primary antibody
Phospho-STAT3 (Ser727)	Rabbit IgG	-	-	9134 S	21	Cell Signaling	1:1000	Primary antibody
STAT3	Mouse IgG2a	-	124H6	9139 S	10	Cell Signaling	1:1000	Primary antibody
TBP	Rabbit IgG	-	D5C9H	44059 S	1	Cell Signaling	1:2000	Primary antibody
UCP1	Rabbit IgG	-	-	ab10983	GR31884 78-5	Abcam	1:1000	Primary antibody
α -TUBULIN	Mouse IgM	-	-	sc-8035	-	Santa Cruz	1:1000	Primary antibody
-	Rabbit IgG	-	-	A6154	SLBG72 01V	Sigma-Aldrich	1:5000	Secondary antibody
-	Mouse IgG	-	-	A4416	SLBS101 0V	Sigma-Aldrich	1:5000	Secondary antibody

2.16 Oil Red O staining & imaging

Oil Red O (Sigma-Aldrich O0625) was used to stain adipocytes after differentiation according to established Lonza protocol (#WEB-PR-PT-2501 OIL-3). Wells were kept covered with water until visualized by bright-field microscopy using a Zeiss AxioObserver A1 Microscope at the VCU Department of Anatomy and Neurobiology Microscopy Facility.

2.17 Lipid droplet quantification

Lipid droplet size was quantified using MIPAR Image Analysis Software. A customized recipe was developed to invert the color of the images obtained from Oil Red O staining and then calculate the area of each lipid droplet via color detection.

2.18 Human subjects research

All human subjects research was performed in accordance with VCU IRB and PRMC-approved protocols (HM20009089 and MCC-17-13470, respectively). Please refer to Appendices for detailed documentation. Below is a summary for of the two aims of this project:

2.18.1 Chart review

Aim: To identify patients with cancer and positive and negative brown adipose tissue on PET imaging and obtain pertinent preliminary information including MRN, zip code, age, gender, ethnicity, BMI, weight, primary cancer, diabetes status, fasting

glucose data, thyroid function testing results, medication list, PET scan image and year, and brown adipose tissue status.

Methods: Investigators will query medical records using Cerner and Montage programs for patients who are at least 40 years of age and who have cancer and have had a PET scan. The PET report will be studied to assess brown adipose tissue presence. Information to be recorded includes: medical record number (MRN), zip code, age, gender, ethnicity, BMI, weight, primary cancer, diabetes status, fasting glucose data, thyroid function testing results, medication list, brown adipose tissue status (either positive or negative depending on PET image report), and PET scan image and year.

For preliminary findings included in Chapter 6, Montage program was queried using the following parameters:

1. All these words: "Brown fat" | "Brown adipose tissue"
2. Date range: 1/1/2007 to 1/31/2017
3. Modalities: PET (PT) and PT/CT

2.18.2 Measuring energy expenditure using whole room indirect calorimeters

Aim: To characterize and measure the difference in energy metabolism profiles, quality of life, and biochemical profiles of patients with cancer with and without evidence of brown adipose tissue activation both at room temperature and following exposure to warm temperature.

Methods: We will recruit patients with cancer and brown adipose tissue activation to compare their energy expenditure with the energy expenditure of age-, sex-, BMI-, and cancer type-matched patients with no brown adipose tissue activation at room

temperature. We will then assess if exposure to warm temperature, a known intervention to quench brown fat activation, is sufficient to decrease the energy expenditure in cancer patients with evidence of BAT activation. The primary endpoint in this Aim will be Energy Expenditure, measured using whole-room indirect calorimeters. The indirect calorimeter technique allows for non-invasive real-time recording of energy expenditure and respiratory quotient (a proxy for substrate utilization) by measuring oxygen consumption and carbon dioxide production. Other data that will be collected for secondary endpoints include: a quality of life questionnaire, thermal comfort questionnaires, infrared photography, blood collection, and urine collection.

There will be three visits in total for participants that complete this study. Visit 1 will be a Screening visit, where informed consent will be obtained, inclusion/exclusion criteria will be confirmed, medical history will be obtained, physical examination will be performed, weight and vital signs will be recorded, bio-impedance analysis will be conducted, and a quality of life questionnaire will be filled out by the participant. At both visits 2 and 3, participants will have their resting energy expenditure measured by resting in the indirect calorimeters for 4 hours per visit. Urine and blood will be collected before and after each session in the calorimeter. Thermal comfort questionnaires will be completed before and after each session in the calorimeter. A general overview of these three visits is visualized in Figure 13.

Detailed methodology regarding calculation of energy expenditure recording is detailed in our laboratory's recently published article, DOI [10.1371/journal.pone.0193467](https://doi.org/10.1371/journal.pone.0193467).

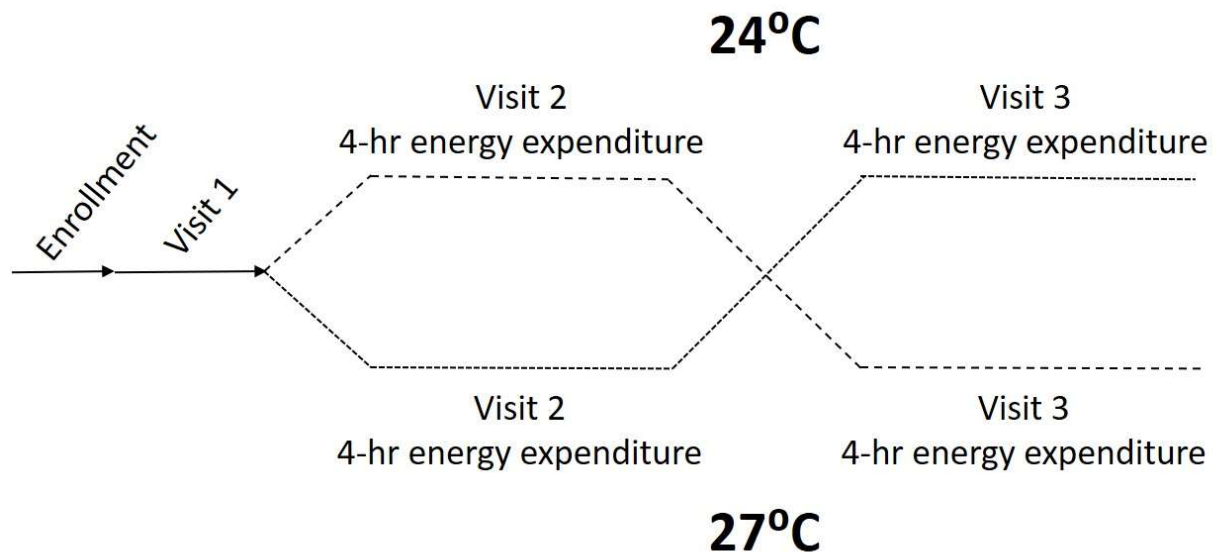


Figure 13. Human subjects research intervention study design

For additional details, refer to Appendices.

2.19 Paraganglioma clinical and laboratory research

2.19.1 Clinical results

Imaging and lab results and clinical notes were obtained without any personal identifiers from the VCUHS Cerner medical system.

All anti-GAD65 antibody assays were performed by Lab Corp using an FDA-cleared enzyme-linked immunosorbent assay (ELISA) purchased from Kronus (KR7710).

2.19.2 Specimen collection

Slides were retrospectively identified archival tissues processed for routine surgical pathology specimen processing in the VCUHS CLIA-compliant clinical lab. Tissues were formalin-fixed and paraffin-embedded. Tissue blocks were cut at 4 micrometers for slide preparation and were deidentified.

2.19.3 Immunohistochemistry and imaging

Hematoxylin and eosin (H&E) staining was performed by the Department of Pathology using routine processing procedures. Human GAD65 protein expression (Santa Cruz, sc-377145) was detected using the SuperPicTure Polymer Detection Kit (Invitrogen 87-8963) and slight protocol modifications. Expression of FH and SDHB was assessed (FH: Santa Cruz sc-100743, SDHB: Abcam ab14714) under routine CLIA-certified clinical protocols as reported previously [163]. Slides were digitally scanned using the NanoZoomer 2.0-HT Whole Slide Imager Digital Pathology Slide Scanner (Meyer Instruments) by the Department of Pathology.

2.20 Statistical analyses

GraphPad Prism was used for visualization of data and statistical analyses. Appropriate tests were used to correct for multiple analyses with nonparametric assumptions; specific tests used are described in each figure legend. For all analyses unless otherwise noted, error bars = standard deviation, and p-values are represented as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; # $p < 0.05$.

Chapter 3: Local Browning of White Adipose Tissue Occurs in Breast Cancer

3.1 Rationale

Browning of WAT has been demonstrated in several murine cancer models and human cancer samples [103, 112, 113]; however, WAT browning in breast cancer has not been established. Breast cancer represents a unique and advantageous model for investigating WAT browning as it is directly contiguous with surrounding breast WAT and is removed from confounding variables such as nausea and malabsorption in gastrointestinal malignancies. In this chapter, we sought out to determine whether WAT browning occurs in breast cancer in both murine breast cancer models and in human breast cancer samples.

3.2 Results

3.2.1 WAT browning occurs locally in the C57BL/6J MMTV-PyVT murine model of spontaneous mammary tumor

We first addressed whether WAT browning could be observed in the C57BL/6J MMTV-PyVT murine model of spontaneous breast cancer. Compared to their respective ovarian WAT (Figure 14A), adipocytes surrounding mammary tumors in both axillary and inguinal mammary fat pads demonstrate an increased staining intensity for UCP1 protein (Figure 14B), but not as much as their respective interscapular BAT (Figure 14A). The lipid droplets in the adipocytes closest to the tumor also appear smaller than lipid droplets further away.

Results from blinded qualitative scoring demonstrate that UCP1 staining intensity of adipose tissue at the tumor-adipose interface is significantly increased (Figure 15A).

Staining intensity of adipose tissue further from the tumor was less than at the tumor-adipose interface in both axillary and inguinal fat pads (Figure 15B). Interestingly, axillary adipose tissues had significantly higher staining intensity further from the tumor compared to inguinal adipose tissues (Figure 15B). The staining intensity gradient, identified as the intensity at the interface minus the intensity further from the tumor, was higher in the inguinal adipose tissues compared to axillary, but not significantly (Figure 15C).

MATLAB analyses on the images with tumor-adipose tissue interface were performed to quantify the number of brown pixels, as a measure of UCP1 staining intensity, across each image. Similar to the results in Figure 15, UCP1 protein expression is most robust at the tumor-adipose tissue interface, and UCP1 protein expression sharply declines moving further from the tumor (representative analysis in Figure 16, with all analyses in Figure 17).

We then looked at gene expression levels of several key adipocyte markers in these adipose tissue depots. Compared to ovarian WAT controls, BAT expressed over 256-fold more *Ucp1* mRNA, while axillary WAT expressed roughly 2-fold more *Ucp1* mRNA and inguinal WAT expressed roughly 8-fold more *Ucp1* mRNA (Figure 18A). For other markers related to thermogenic adipose tissue, interscapular BAT, compared to ovarian WAT control, expressed significantly more *Cidea* (cell death-inducing DNA fragmentation factor, alpha subunit-like effector A), increased (though not significantly) *Oplah* (5-oxoprolinase), *Ppargc1a* (peroxisome proliferative activated receptor, gamma, coactivator 1 alpha), and *Slc29a1* (equilibrative nucleoside transporter 1), and no change or less *Pparg* (peroxisome proliferative activated receptor, gamma) and *Slc27a1*

(long-chain fatty acid transport protein 1) (Figure 18B). Expression of these genes was more variable for the axillary and inguinal WAT with tumor; although there were no statistically significant increases in gene expression, many of the WAT depots had increased expression of thermogenic adipose tissue genes compared to ovarian WAT control. For example, the right inguinal WAT depots expressed an average 3-fold more *Cidea* and *Slc29a1*, and axillary WAT depots demonstrate an average 20-fold increase in expression of *Opfah*, and 3-fold increase in expression of *Slc29a1* (Figure 18B). Lastly, both *Lipe* (hormone sensitive lipase) and *Lpl* (lipoprotein lipase) expression levels were significantly decreased in WAT with tumor compared to ovarian WAT control (Figure 18C).

We plotted individual values along with the average expression value for each adipose tissue and gene tested, which demonstrate a large range of variability in expression values for each adipose tissue depot. (Figure 19 panels B, D, and E).

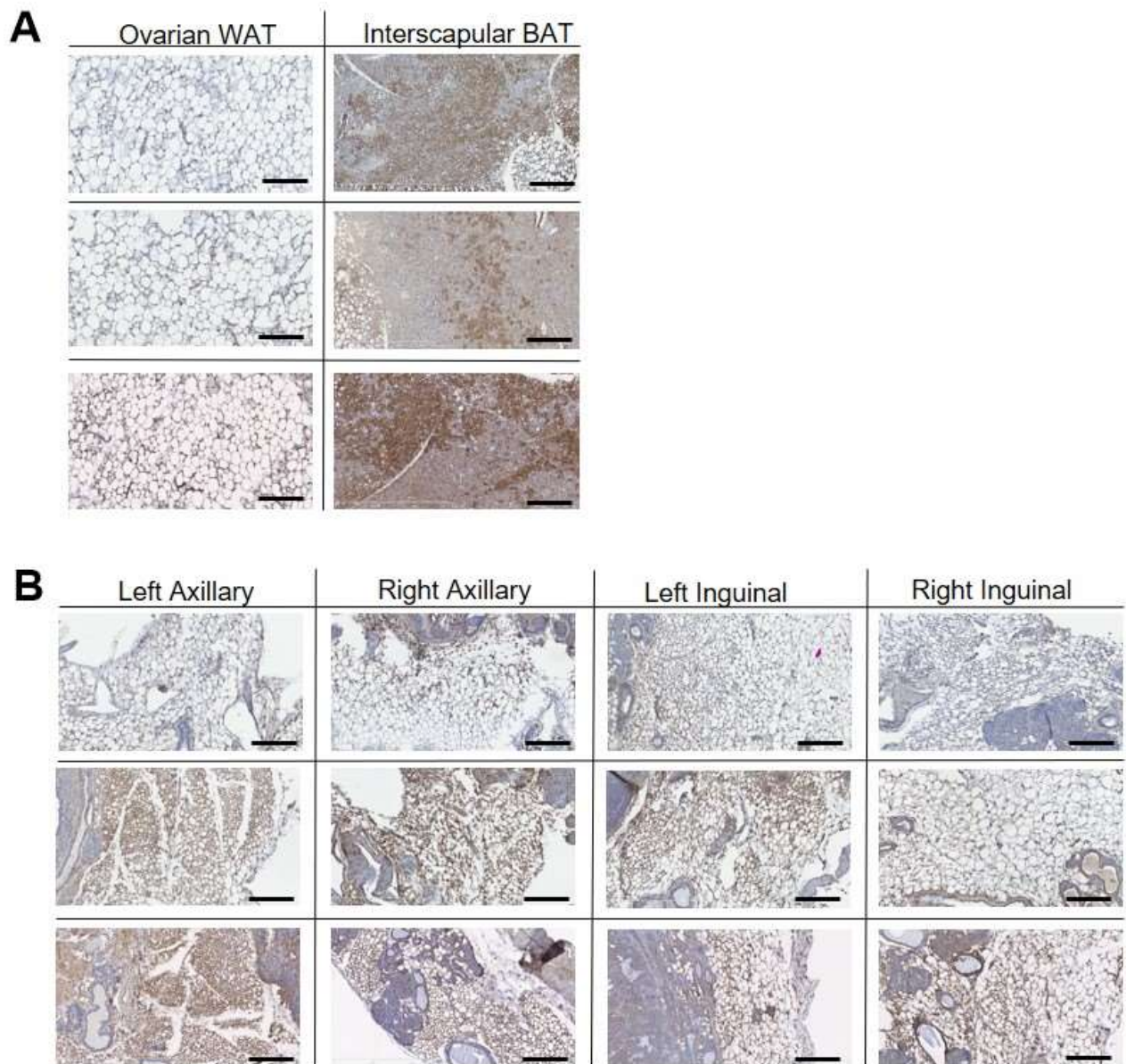


Figure 14. Immunohistochemistry shows increased UCP1 protein expression in adipocytes adjacent to spontaneous mammary tumors

Four-month old female C57BL/6J MMTV-PyVT mouse tissue was harvested and stained for UCP1 protein in (A) ovarian WAT (negative controls), interscapular BAT (positive controls), and (B) axillary and inguinal mammary fat pads with tumor. Each row corresponds to one of three individual mice. Scale bars = 200µm. UCP1 = uncoupling protein 1, WAT = white adipose tissue, BAT = brown adipose tissue.

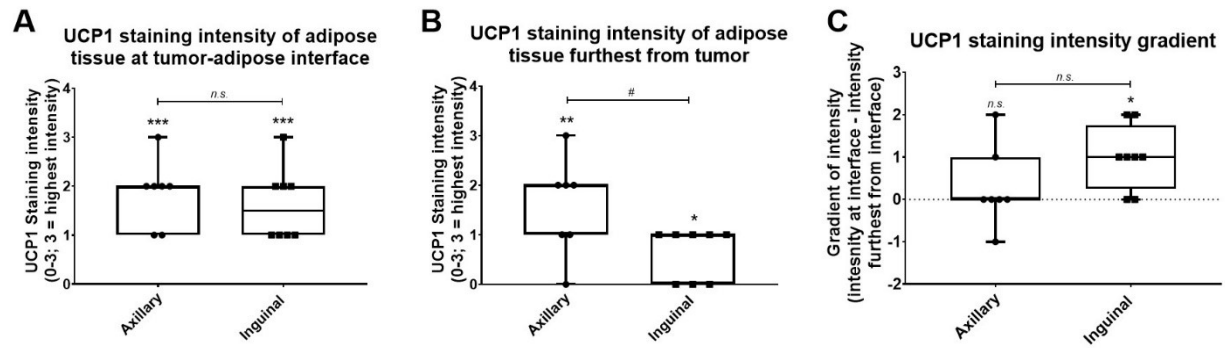


Figure 15. Qualitative scoring of UCP1 staining intensity in adipocytes adjacent to spontaneous mammary tumors

Scoring was performed in adipocytes at (A) the tumor-adipocyte interface and (B) further from tumor, as well as staining intensity gradient (C). Statistical tests: (A), (B), and (C) one sample t-tests for each WAT depot compared to null hypothesis of no staining; unpaired t-tests to compare staining intensity between axillary and inguinal WAT depot. UCP1 = uncoupling protein 1.

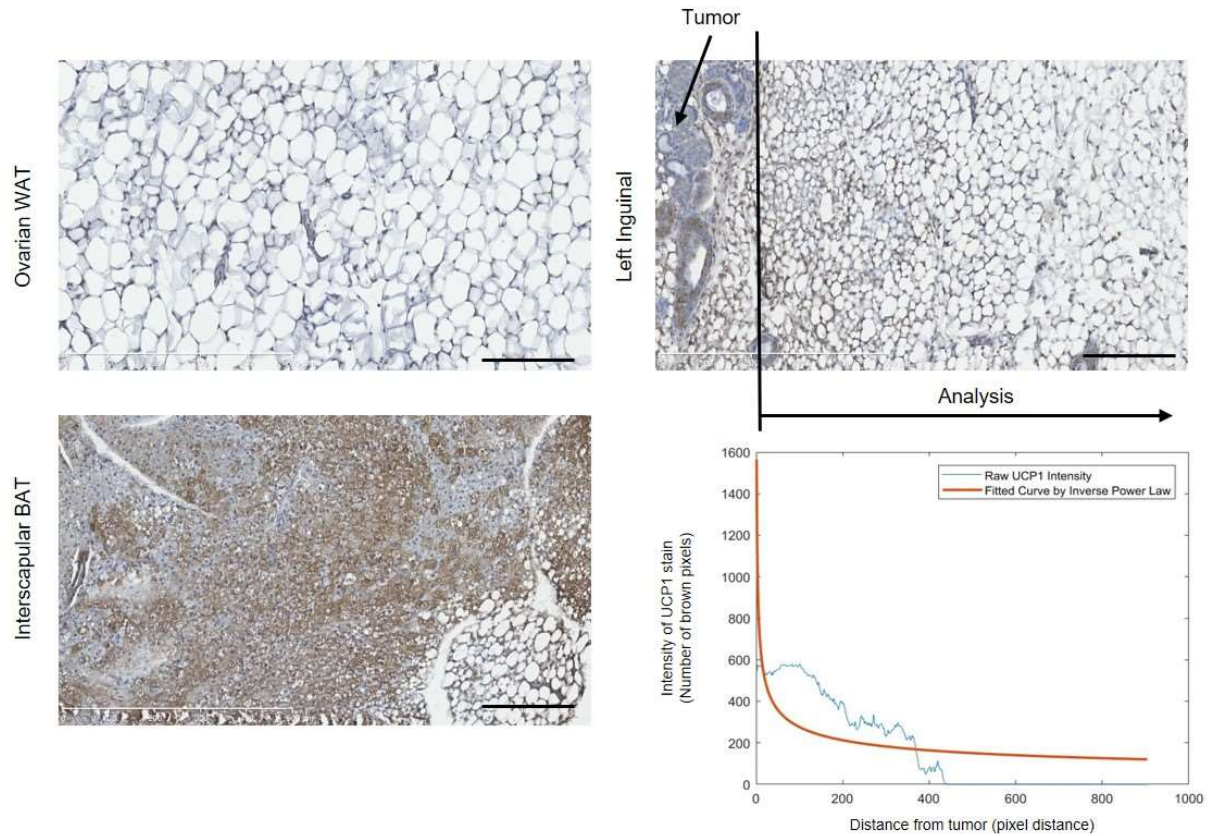


Figure 16. Representative MATLAB analysis of immunohistochemistry for UCP1 in adipocytes adjacent to spontaneous mammary tumors

Results indicate a sharp decline in UCP1 staining intensity with increasing distance from the tumor. Scale bars = 200µm. UCP1 = uncoupling protein 1, WAT = white adipose tissue, BAT = brown adipose tissue.

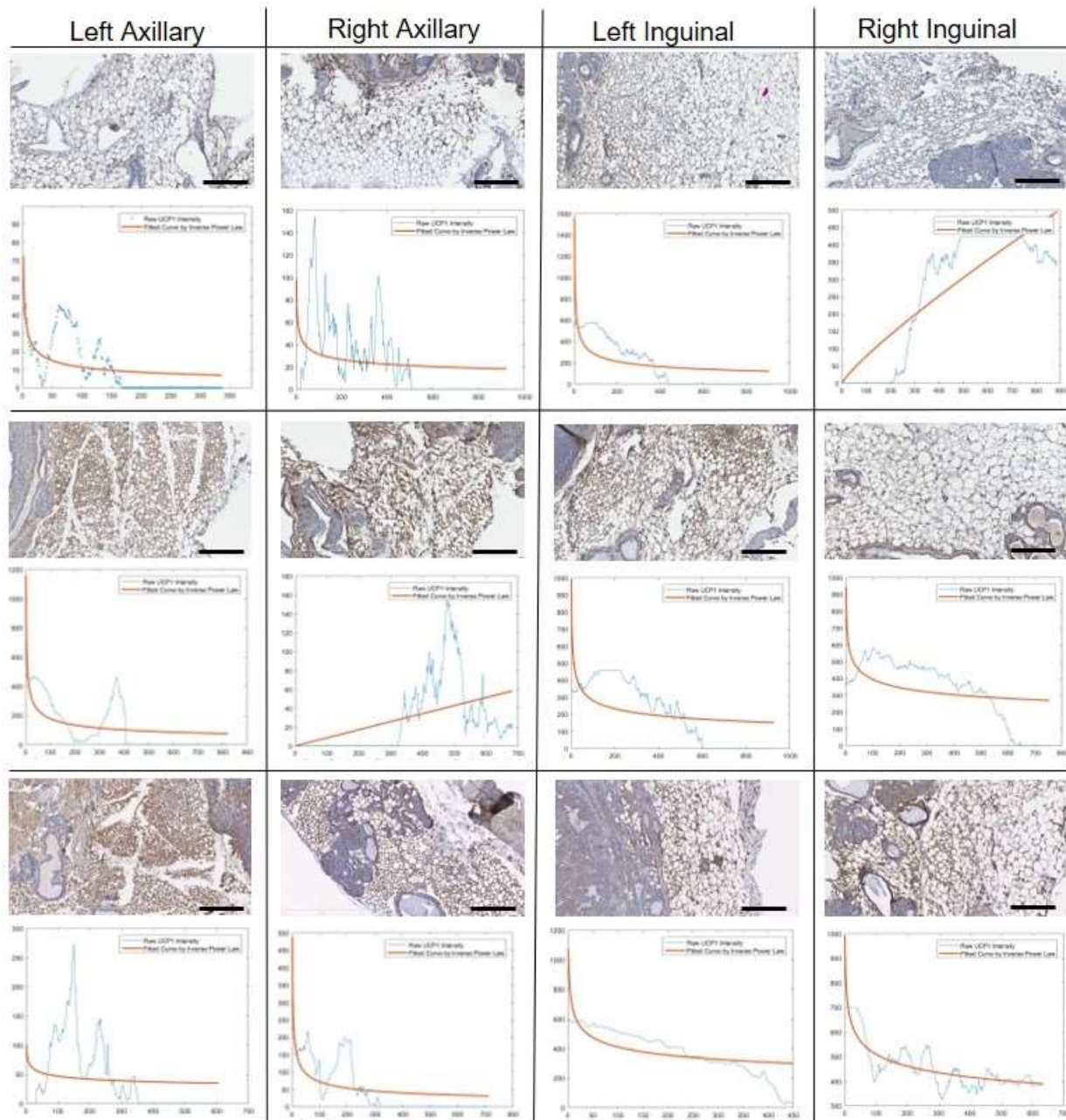


Figure 17. MATLAB analyses of UCP1 immunohistochemistry in adipocytes adjacent to spontaneous mammary tumors

Indicating a sharp decline in UCP1 staining intensity with increasing distance from the tumor. Scale bars = 200µm. UCP1 = uncoupling protein 1.

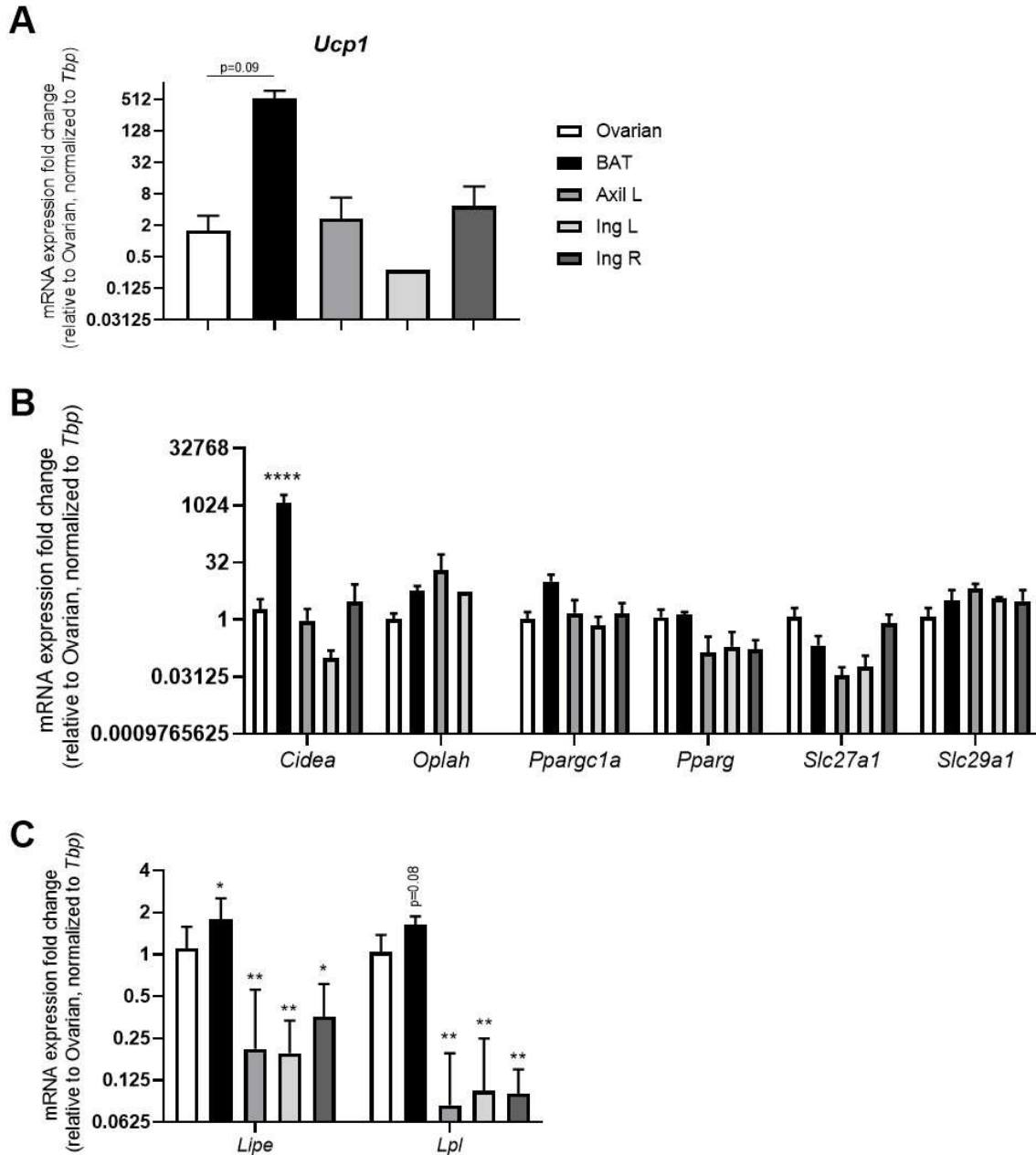


Figure 18. Adipose tissue depots contiguous to spontaneous mammary tumors have altered expression of several adipocyte-related genes

Four-month old female C57BL/6J MMTV-PyVT mouse tissue was harvested for qPCR in ovarian WAT ('Ovarian' in image legend; negative controls), interscapular BAT ('BAT' in image legend; positive controls), left axillary WAT ('Axil L' in image legend), and left and right inguinal WAT ('Ing L' and 'Ing R' in image legend, respectively). Quantitative PCR for (A) *Ucp1*, (B) markers of thermogenic adipose tissue, and (C) other markers of white adipocytes and WAT lipolysis. Please note y-axis scale is log2. n = 2-4 mice. Statistical tests: (A) Kruskal-Wallis and Dunn's multiple comparisons tests, (B) and (C) Two-way ANOVA and Dunnett's multiple comparisons tests.

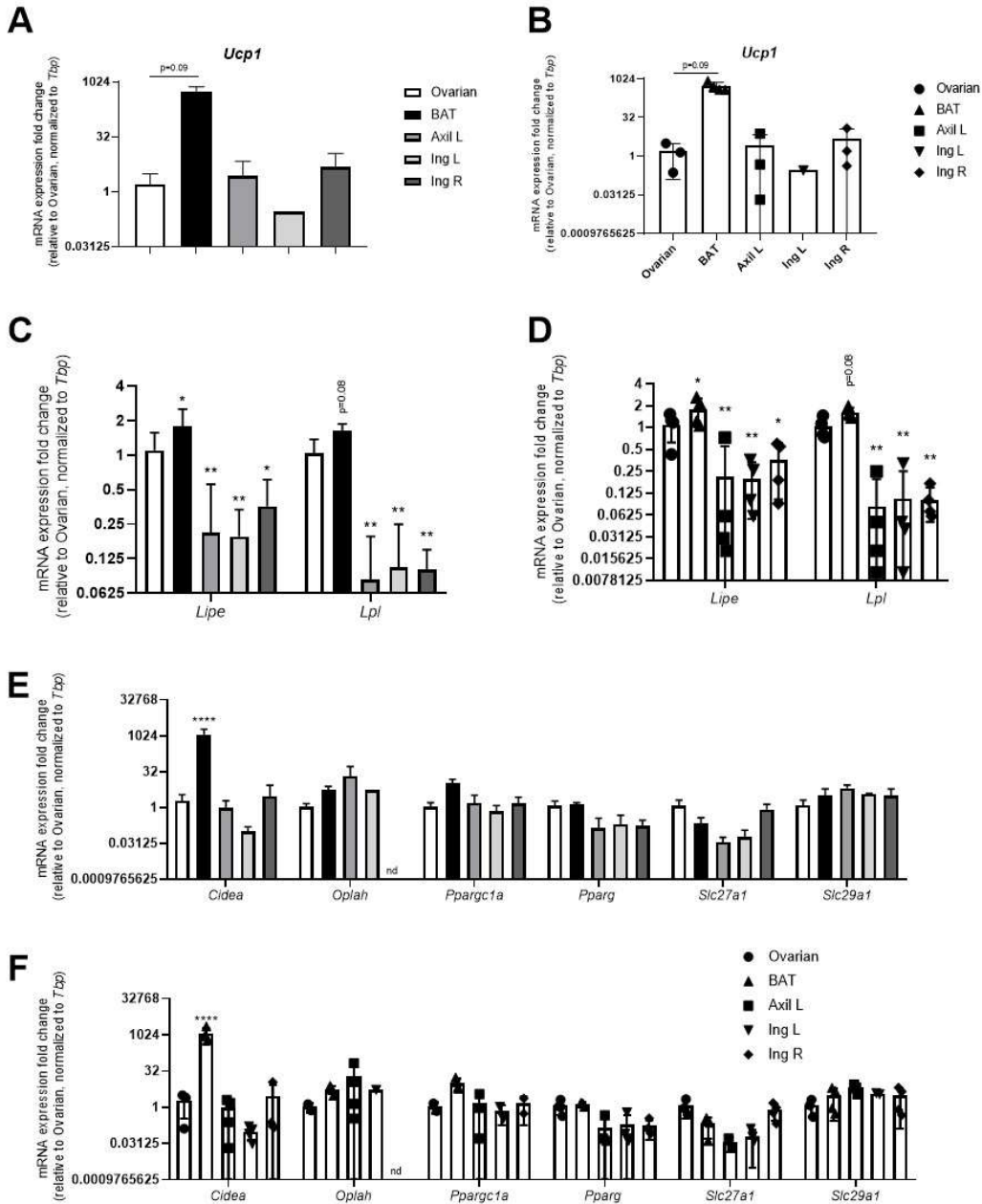


Figure 19. Variability of gene expression changes in white adipose tissue depots contiguous to spontaneous mammary tumors

WAT ('Ovarian' in image legend; negative controls), interscapular BAT ('BAT' in image legend; positive controls), left axillary WAT ('Axil L' in image legend), and left and right inguinal WAT ('Ing L' and 'Ing R' in image legend, respectively). (A), (C), and (E) are the same from Figure 15, (B), (D), and (F) show same averages but with individual data points plotted. Please note y-axis scale is log2. n = 2-4 mice. Statistical tests: (A) and (B) Kruskal-Wallis and Dunn's multiple comparisons tests, (C), (D), (E), and (F) Two-way ANOVA and Dunnett's multiple comparisons tests.

3.2.2. *WAT browning occurs with time specificity in the FVB MMTV-PyMT murine model of spontaneous mammary tumor*

We next asked whether WAT browning could be observed in another murine mouse model of spontaneous breast cancer, FVB MMTV-PyMT, at the protein level. Western blot for UCP1 was performed at two timepoints, 4 weeks and 13 weeks, for both wild type mice and mice with spontaneous mammary tumor development (referred to as 'PyMT mice'). Image of western blot is included in Figure 20A. We quantified the blot intensity for each group using Image J software (Figure 20B,C). Compared to wild-type (WT) age-matched controls, PyMT mice demonstrate increased UCP1 protein expression in inguinal adipose tissue at 4 weeks of age; interestingly, this trend is reversed at 13 weeks (Figure 20B). In interscapular BAT, UCP1 expression is increased in PyMT mice at 13 weeks, but not at 4 weeks (Figure 20C).

Gene expression analysis on the adipose tissue pads in PyMT mice demonstrates an approximate 2-fold decrease in *Ucp1* mRNA expression at 13 weeks compared to 4 weeks in both inguinal WAT (Figure 21A) and interscapular BAT (Figure 21B). Figure 21C demonstrates that at both timepoints, interscapular BAT expresses at least 50-fold more *Ucp1*.

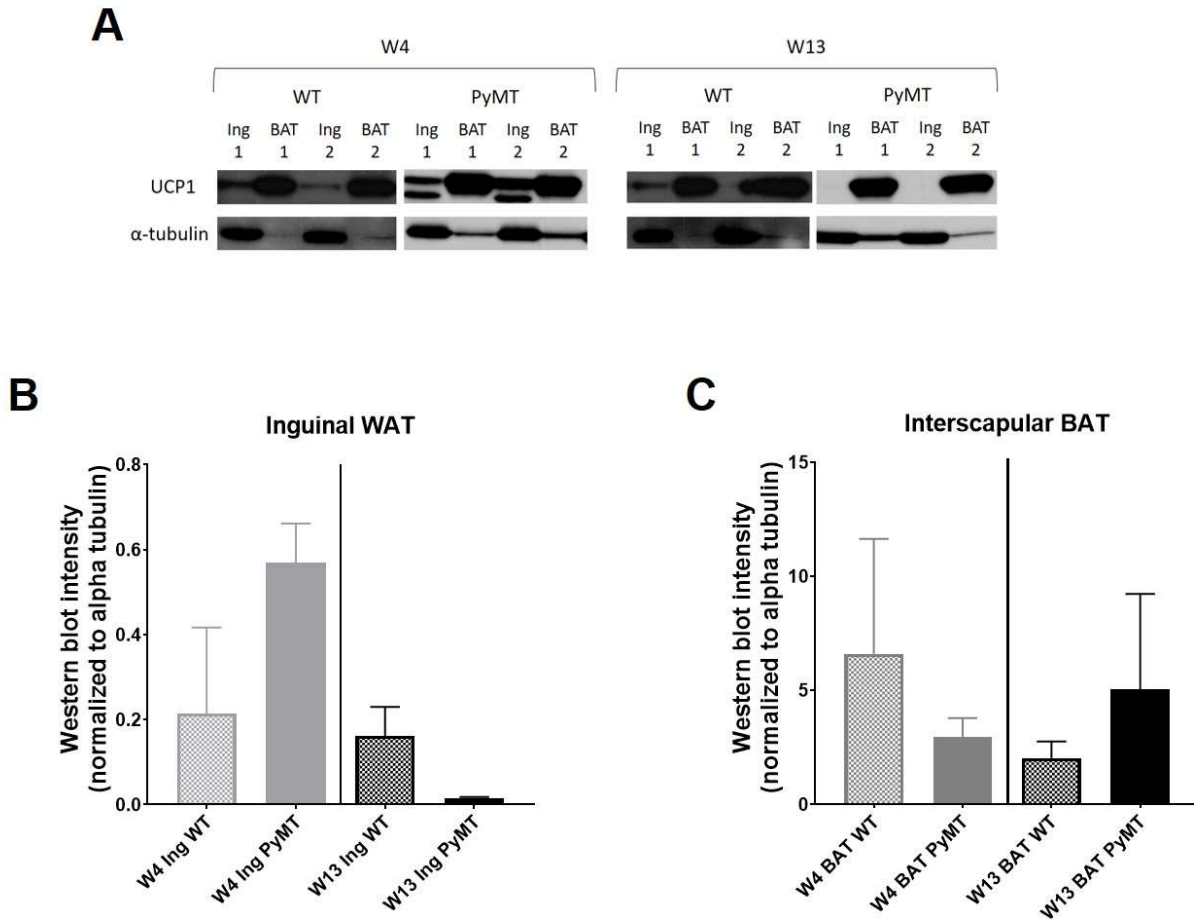


Figure 20. Adipose tissue depots in mice with spontaneous mammary tumors have time-dependent changes in UCP1 protein expression

Four and thirteen week-old female FVB MMTV-PyMT with age-matched wild-type (WT) tissue was harvested for protein analysis in interscapular BAT ('Interscapular BAT' or 'BAT' in image legend; positive controls), and inguinal WAT ('Inguinal WAT' or 'Ing' in image legend, respectively). (A) Western blot of UCP1 and alpha tubulin. Quantification of western blot for (B) inguinal WAT and (C) interscapular BAT.

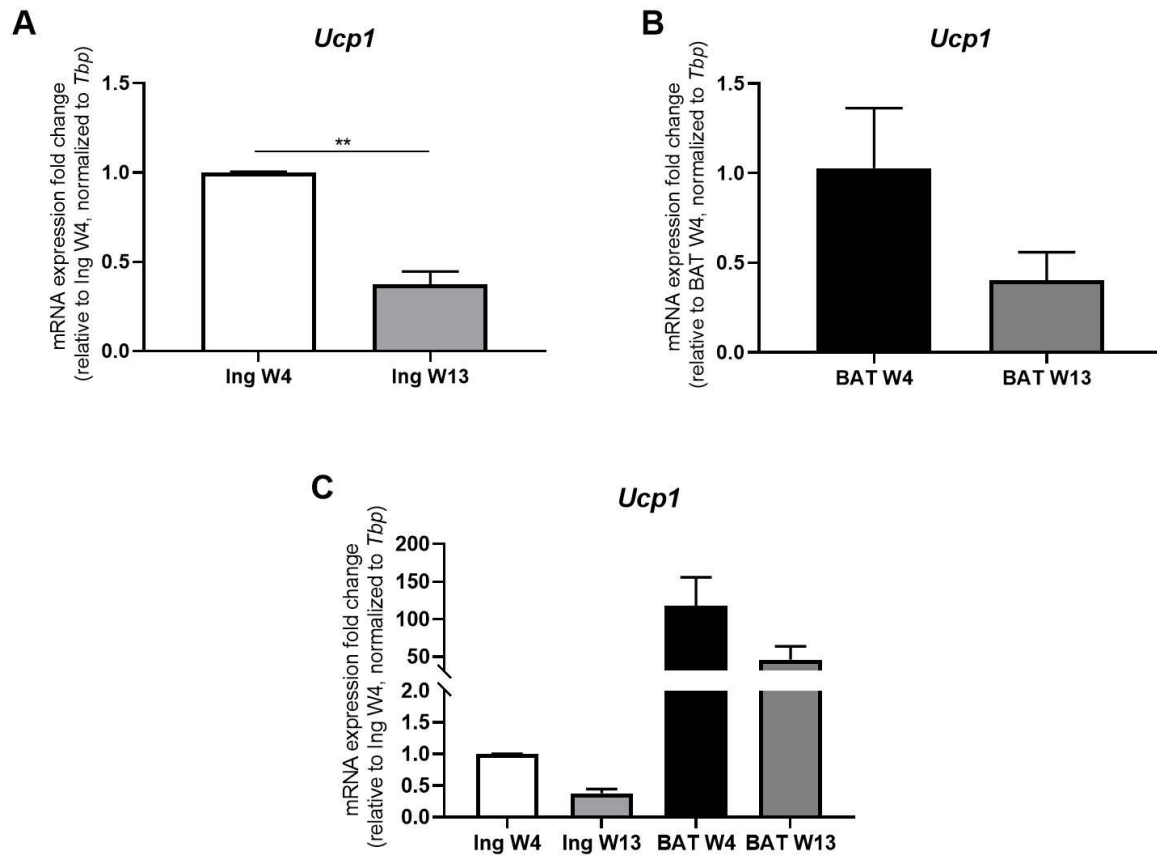


Figure 21. Adipose tissue depots in mice with spontaneous mammary tumors have time-dependent changes in *Ucp1* mRNA expression

Four and thirteen week-old female FVB MMTV-PyMT was harvested for gene expression analysis in interscapular BAT ('Interscapular BAT' or 'BAT' in image legend; positive controls), and inguinal WAT ('Inguinal WAT' or 'Ing' in image legend, respectively). Quantitative qPCR for (A) *Ucp1* in inguinal WAT and (B) *Ucp1* in interscapular BAT with results normalized to respective 4-week expression levels. (C) *Ucp1* mRNA expression for both adipose tissue depots, normalized to 4-week inguinal WAT.

3.2.3. Adipose tissue adjacent to human breast cancer exhibits variable WAT browning

Lastly, we explored whether white adipose tissue adjacent to human samples of breast cancer exhibits browning. We first obtained samples of benign breast WAT from elective breast reduction surgeries to serve as our negative controls, and stained them for UCP1. Breast WAT exhibits no to minimal UCP1 protein expression, and lipid droplet sizes are larger – both features we expect from benign WAT (Figure 22).

We then stained human breast cancer samples with adjacent adipose tissue for UCP1. These breast cancer samples were previously identified by the TDAAC and/or Department of Pathology as either triple negative, estrogen receptor positive, or HER2 expressing. For all breast cancer types, adipose tissue at the tumor-adipocyte interface express more UCP1 than benign breast WAT, and in many samples, have lipid droplets that are smaller in diameter compared to benign breast WAT (representative images, one from each cancer type in Figure 23). While expression of UCP1 appears to be greater than benign breast WAT in all samples, the intensity of stain appears to vary significantly (Figure 24, Figure 25, Figure 26).

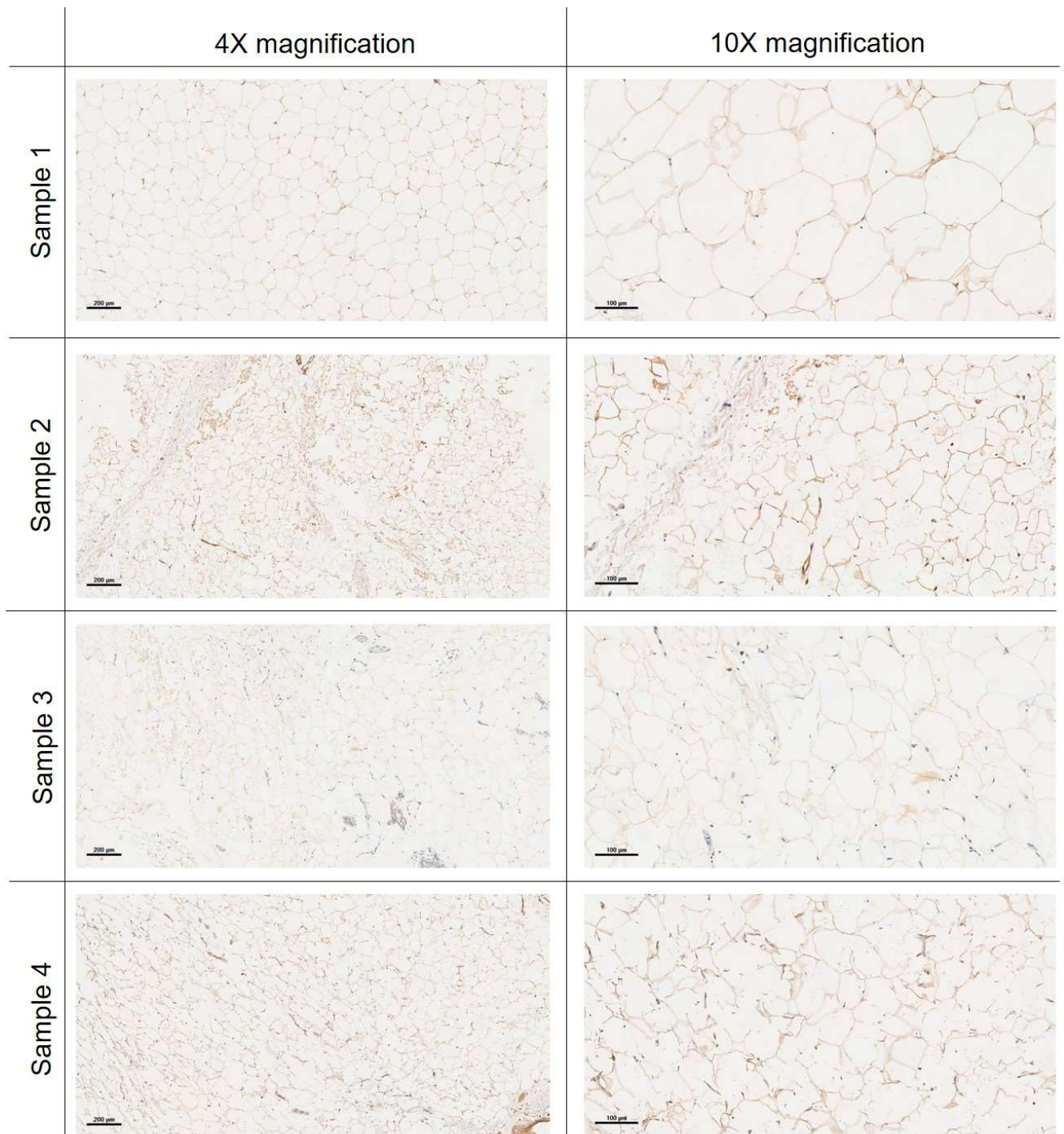


Figure 22. Benign breast white adipose tissue from human female subjects do not express UCP1 protein

Breast WAT from four different female subjects undergoing elective breast reduction surgery was stained for UCP1 protein. Each row corresponds to one of four individual human subjects. Scale bars for 4X magnification = 200µm; 10X magnification = 100µm.

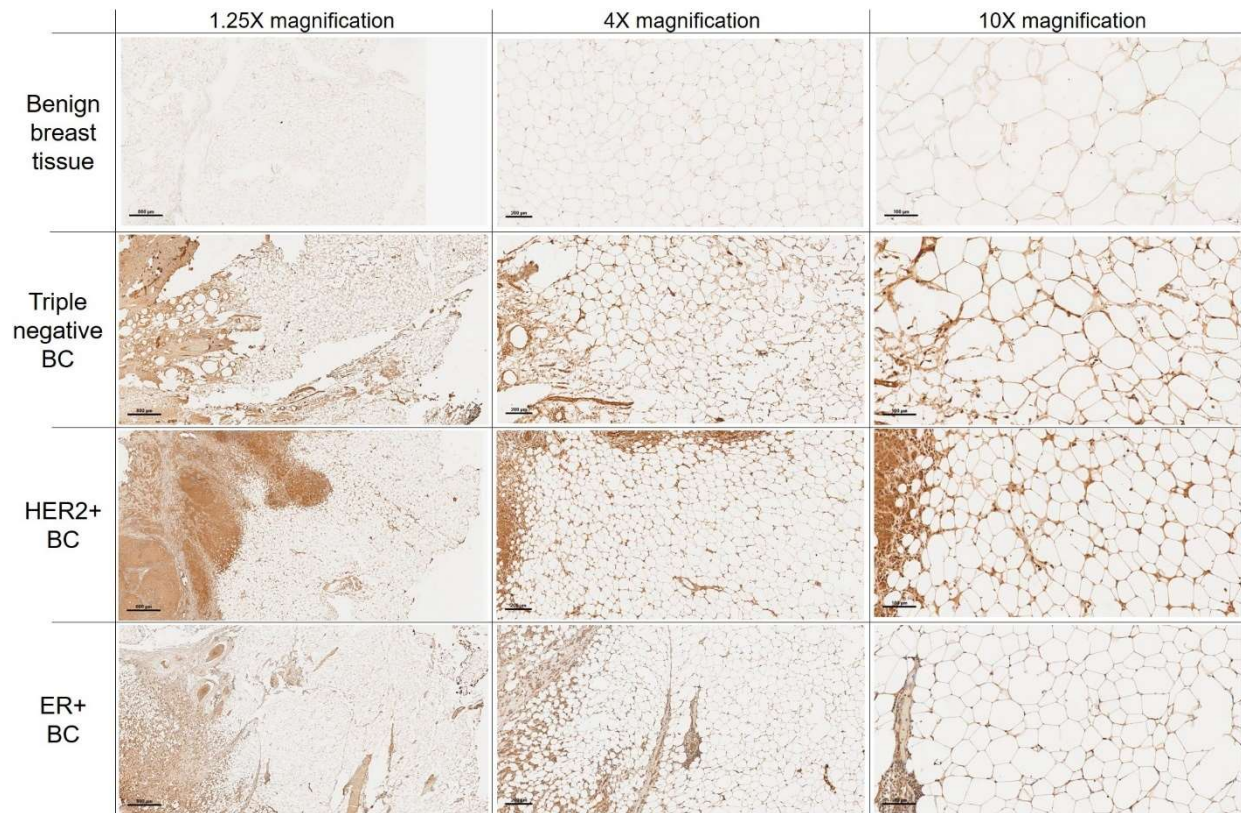


Figure 23. Immunohistochemistry shows increased UCP1 protein expression and decreased lipid droplet size in adipocytes adjacent to human breast carcinomas

Representative IHC images for UCP from the following: Benign breast tissue (1st row), triple negative breast cancer (2nd row), HER2+ breast cancer (3rd row), estrogen receptor breast cancer (ER+ BC, 4th row). Scale bars for 1.25X magnification = 800µm; 4X magnification = 200µm; 10X magnification = 100µm.

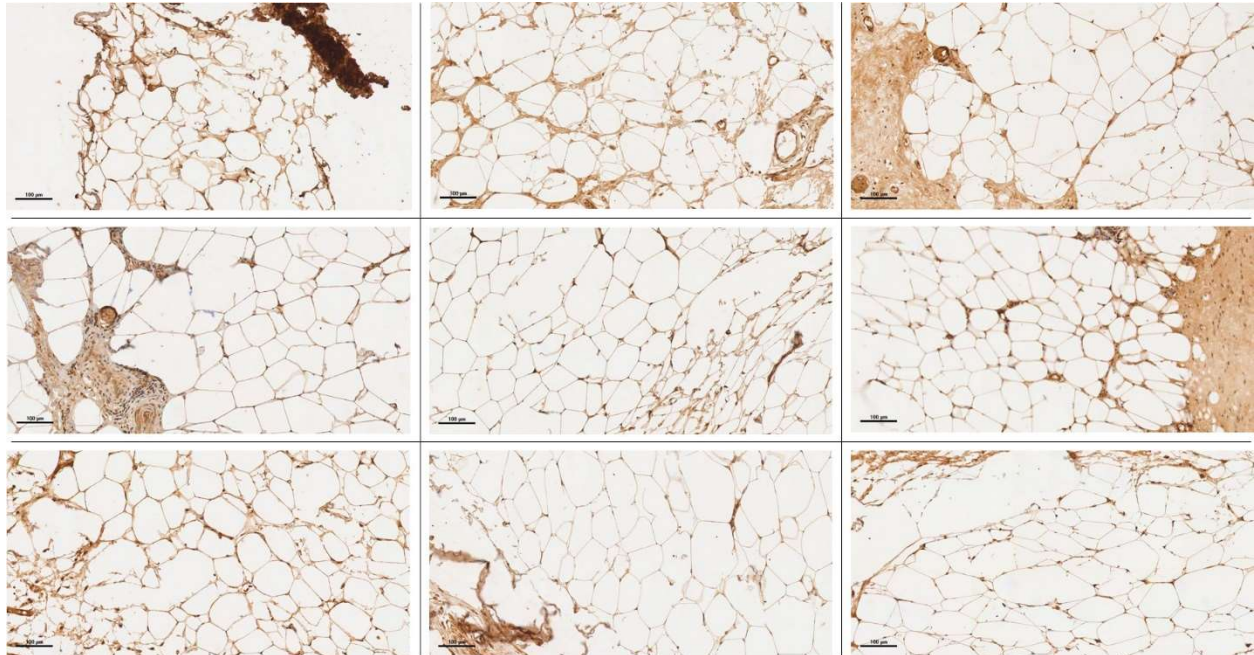


Figure 24. Immunohistochemistry shows variability in UCP1 expression in adipocytes adjacent to triple negative human breast carcinomas.

Each image represents a unique patient sample. All images at 10X magnification, with scale bars = 100µm.

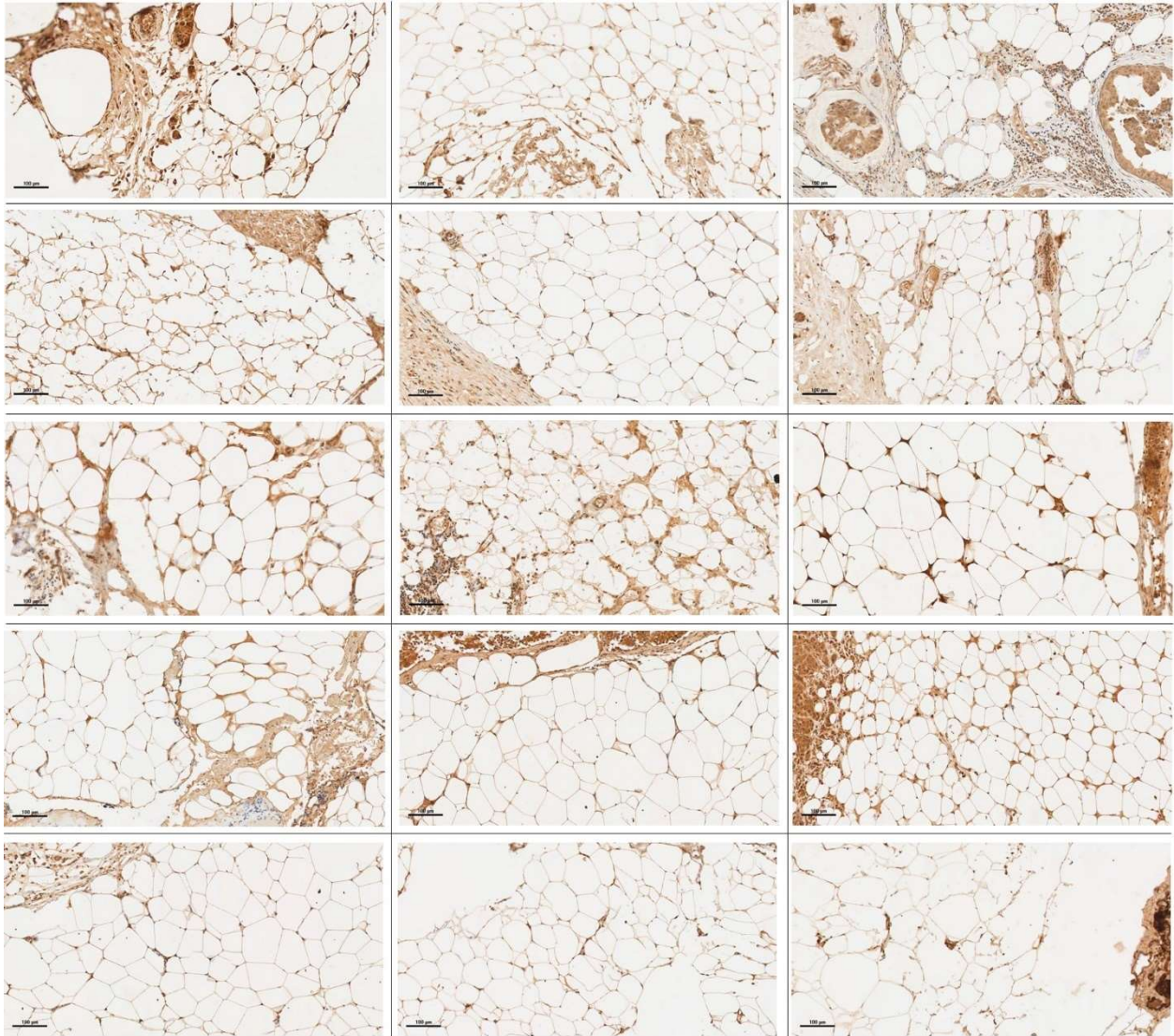


Figure 25. Immunohistochemistry shows variability in UCP1 expression in adipocytes adjacent to HER2+ human breast carcinomas.

Each image represents a unique patient sample. All images at 10X magnification, with scale bars = 100µm.

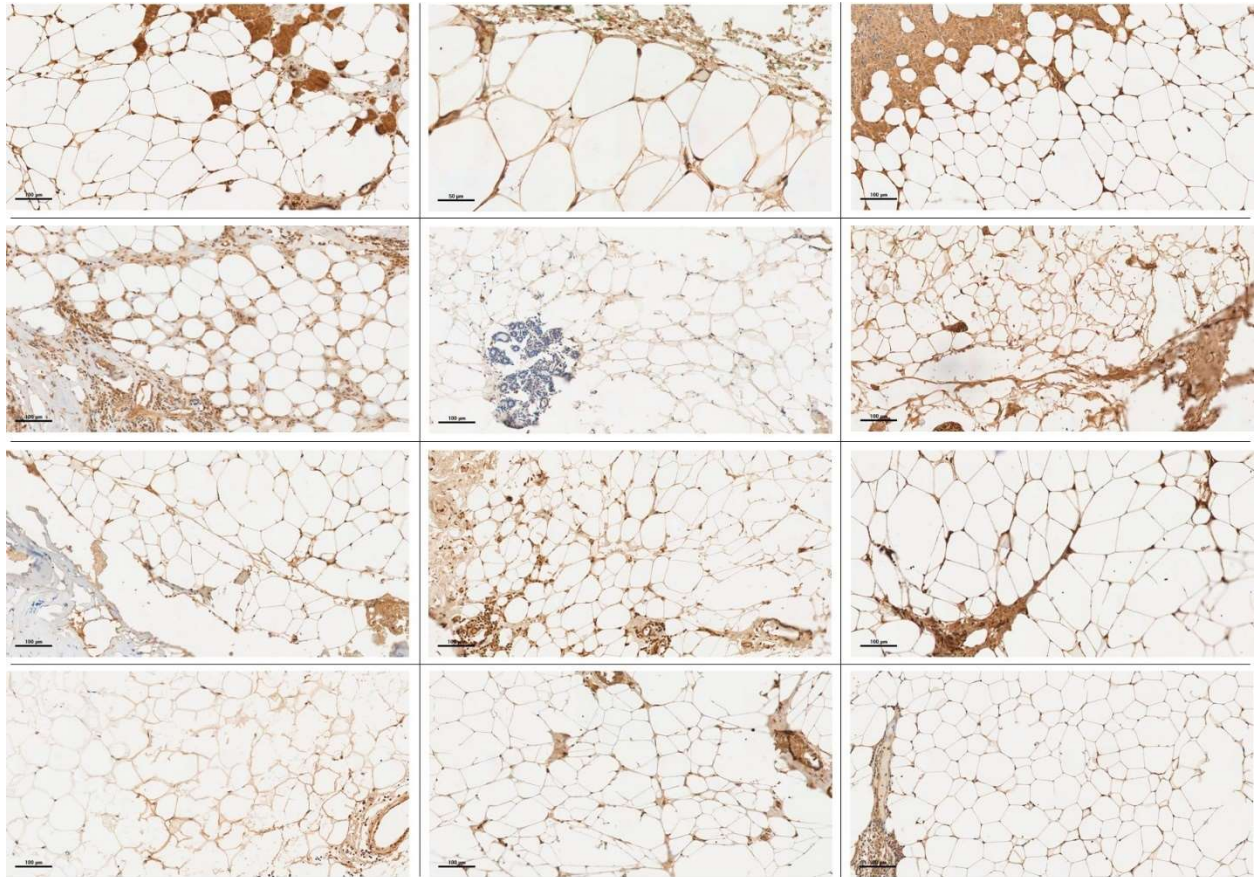


Figure 26. Immunohistochemistry shows variability in UCP1 expression in adipocytes adjacent to estrogen receptor-positive (ER+) human breast carcinomas.

Each image represents a unique patient sample. All images at 10X magnification, with scale bars = 100µm.

3.3 Discussion

We first asked whether WAT browning could be observed in murine models of breast cancer. In the C57BL/6J MMTV-PyVT model, we show that adipocytes closest to the tumor-adipocyte interface *in vivo* have increased UCP1 protein expression, the hallmark of thermogenic adipose tissue function, via immunohistochemistry (Figure 14). The increase in UCP1 protein expression is most robust at the tumor-adipose tissue interface, and UCP1 protein expression sharply declines moving further from the tumor, demonstrated qualitatively by higher intensity scoring at the tumor-adipocyte interface (Figure 15) and quantitatively by a trend that fits the inverse power law upon curve fitting (Figure 16, Figure 17). This suggests that the strength of browning is inversely related to the center of its driver, the tumor cells, which has recently been noted in a xenograft model of breast cancer as well [117]. We also observe smaller lipid droplets in these adipocytes, which has been observed and described as a characteristic of ‘cancer-associated adipocytes (CAA)’ at the tumor forefront [116]. Together, the decreased lipid size and increased UCP1 expression in adipocytes closest to the tumor in our IHC images confirm the phenotypic changes consistent with a maladaptive transdifferentiation from white to beige adipose tissue in response to the tumor microenvironment. Furthermore, the localized nature of this browning suggests a paracrine signaling interaction between the tumor and adjacent adipocytes.

mRNA expression levels of key adipocyte gene markers further support adipocyte plasticity in these WAT depots with tumor (Figure 18). *Ucp1* mRNA expression was increased in two of the three WAT fat pads with spontaneous mammary tumor, with up to an 8-fold increase in expression. Other markers related to thermogenic

adipose tissue, such as *Cidea*, *Oplah*, and *Slc29a1*, were increased in the axillary and/or inguinal WAT with tumor development. Interestingly, WAT in the fat pads with tumor expressed significantly less *Lipe* and *Lpl*, two markers of WAT lipolysis. This supports the hypothesis that the smaller lipid droplets observed at the tumor-adipocyte interface in our IHC images are the results of WAT browning as opposed to WAT lipolysis.

It is important to highlight the variability in the mRNA expression data, most notably in the thermogenic adipose tissue-related genes (Figure 19). This variability is not surprising considering that the tissue analyzed was comprised of white adipose tissue both proximal and distal to the spontaneous mammary tumors. It is likely that WAT at the tumor-adipocyte interface was variably excised, as it was dissected via manual inspection. We do not have the capability to perform laser capture microdissection on our samples, which would be the best way to assess this, especially considering the very localized (~200um) browning effect noted on IHC.

In another murine model of spontaneous breast cancer, the FVB MMTV-PyMT model, we demonstrate additional evidence to support WAT browning in breast cancer. Interestingly, both WAT and BAT depots in these mice with early (4 week) and later (13 week) stage tumors appear to exhibit time-dependent changes in UCP1 protein expression (Figure 20). Pooled samples of human breast cancer xenografts grown in mice have been shown to express varying amounts of UCP1 protein dependent on the length of xenograft growth, with UCP1 present at 1 week post-implantation, peaking at 5-7 weeks, and decreasing to less than the 1-week level at 15 weeks [117]. While these changes may have contributions from cells within the entire tumor microenvironment,

our results suggest temporal regulation of adipocyte plasticity in breast cancer. *Ucp1* mRNA expression levels in these tumors suggest a decrease in transcript expression at later timepoints in both inguinal WAT and interscapular BAT (Figure 21); however, these results were obtained by comparing mice with tumor, without the use of age-matched controls as in our western data. We also recognize the same limitations to these results as explained for the C57BL/6J MMTV-PyVT mRNA results earlier in this section. Additional studies with a larger samples sample size (as ours only had 2 mice per group) would be useful to confirm this time-dependence, and eventually probe into the mechanisms behind it.

Lastly, as we have seen WAT browning in two different murine models of spontaneous mammary tumor, we asked whether WAT browning occurs in human samples of breast cancer. We first demonstrate that benign breast WAT has no UCP1 expression at baseline using samples from 4 women of different ages (Figure 22), and then show via IHC that UCP1 protein expression is increased in white adipocytes surrounding human breast carcinoma samples (Figure 23). This supports the global concept of WAT browning in cancer, as IHC studies performed by Petruzzelli and colleagues demonstrated that several other human cancers (melanoma, colon, pancreatic, and lung cancers) were associated with increased UCP1 in WAT [112].

In human breast cancer samples, similar to what we observed in our mouse IHC, there appears to be a gradient of UCP1 expression, highest at the tumor-adipocyte interface and decreasing moving distally from the tumor (Figure 23). Interestingly, it appears that some of the tumor cells stain positively for UCP1 in several of the samples. In the literature, there are several reports of UCP1 expression in cancer, including

breast cancer xenografts and human samples of non-small cell lung cancer [117, 164], so this observation is not surprising.

We further demonstrate that this increase in UCP1 expression in WAT in cancer is seen in a variety of subtypes of breast carcinoma, including triple negative (Figure 24), HER2 expressing (Figure 25), and ER+ (Figure 26) breast cancers. Future analyses using additional samples with a larger volume/area of WAT adjacent to tumors will be helpful to determine whether differences exist between receptor statuses. Correlating stage of tumor at time of resection with UCP1 staining intensity would provide additional insight into the time-dependency we noted in our murine studies on the human level.

While increased UCP1 staining intensity is observed in all breast cancer subtypes we analyzed, there is certainly variability in staining intensity between individual samples (Figure 24, Figure 25, Figure 26). It is well established that inter- and intra-organism tumor heterogeneity exists in breast cancer [165, 166]. Since we hypothesize that the tumor and tumor microenvironment are responsible for these adipocyte changes, it is therefore not surprising that the variability between tumor and host responses would result in variable adipocyte changes.

3.4 Conclusion

Taken together, our results demonstrate the presence of WAT browning (increased UCP1 expression and decreased lipid droplet size) in adipose tissue surrounding tumors in both murine and human breast cancers. This illustrates the importance and translational potential of our work. Furthermore, our results suggest a

local, paracrine (cell-cell) interaction between adipocytes and tumor and tumor microenvironment as the driver of the adipocyte changes. These are the interactions that we explore in the next two chapters.

Chapter 4: Cancer-Adipocyte Interactions Induce Changes to White Adipocytes *In Vitro*

4.1 Rationale

Based on the WAT browning observed *in vivo* (Chapter 3), we established a cell culture system which would allow us to methodically and systematically study the effects of individual components of the tumor microenvironment on adipocytes *in vitro*. Furthermore, as the WAT browning demonstrated in Chapter 3 is extremely localized in nature, and other studies have implicated tumor-secreted factors in WAT browning and remodeling [113], we asked whether local factors secreted by breast cancer cells (and other cancer cells) are the driver of these local WAT changes.

4.2 Results

4.2.1 Immortalized murine SVF cells are capable of undergoing differentiation to mature white and beige adipocytes

We first tested the differentiation capacity of our lab's immortalized male mouse inguinal stromal vascular fraction cells (referred to as mouse immortalized white preadipocytes, or mIWPA's). Immature stromal vascular fraction cells are able to successfully undergo differentiation to both mature white and beige adipocytes with high percentage of differentiation (Figure 27A). Expression of *Dlk1* (Delta like non-canonical Notch ligand 1), a marker of immature/undifferentiated adipocytes, is increased by about 5-fold in undifferentiated preadipocytes compared to differentiated white and beige adipocytes (Figure 27B). Expression of other gene markers associated with mature adipocytes was decreased in the preadipocytes, most notable being *Cidea*.

Lastly, *Ucp1* was significantly increased in the mature beige adipocytes, demonstrating that our *in vitro* immortalized cell culture model expresses appropriate differences in thermogenic gene expression.

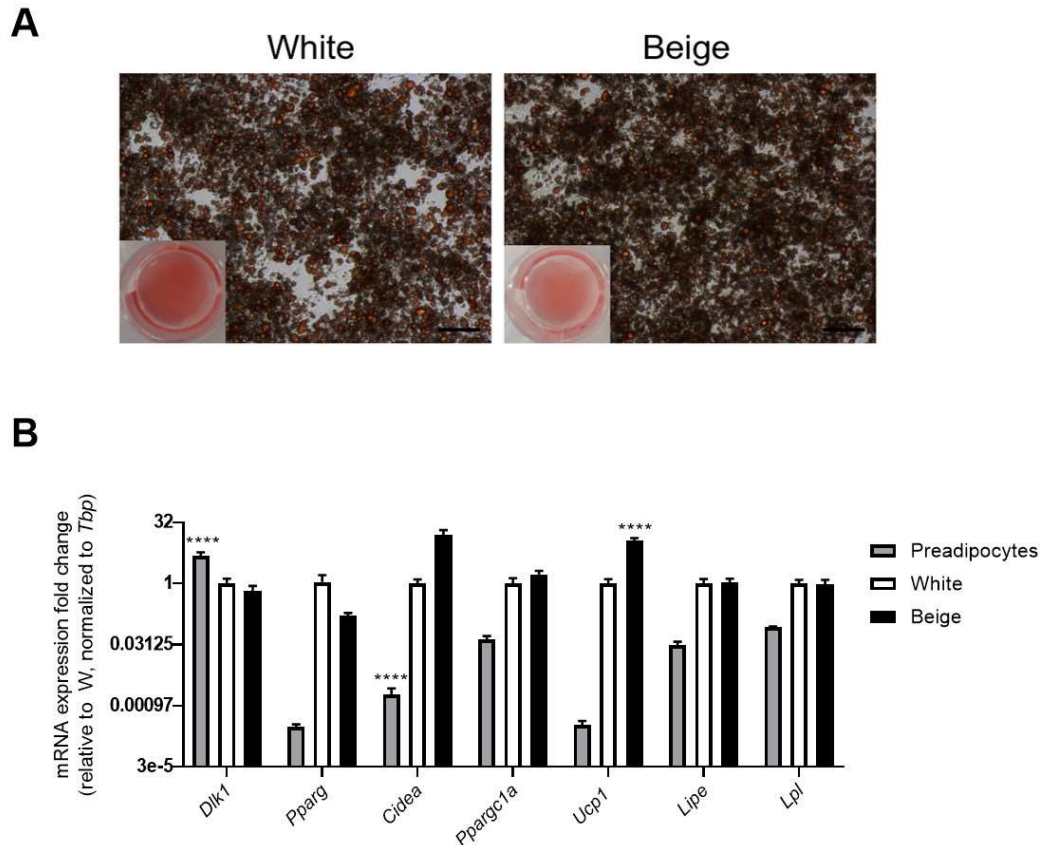


Figure 27. Immortalized murine SVF are capable of differentiation to mature white and beige adipocytes

Inguinal SVF isolated from inguinal WAT from a young male C57BL/6J mouse were immortalized, then cultured and differentiated to either white or beige adipocytes as described in methods. (A) Representative Oil Red O staining after completed induction and maintenance protocol. Scale bars = 100µm. (B) Quantitative PCR for various adipocyte-related markers. Please note y-axis scale is log2. n = 4 for all groups. Error bars = standard deviation. Statistical tests: (B) 2-way ANOVA and Dunn's multiple comparisons tests.

4.2.2 Cancer conditioned medium from E0771 and LLC cells do not induce WAT browning in our immortalized cell culture model

We next asked if white preadipocyte exposure to cancer-conditioned medium would induce maturation of beige adipocytes. As described in methods section 2.8.1 (Figure 7), immortalized preadipocytes were exposed to cancer conditioned medium at various stages of differentiation: either the induction phase, the maintenance phase, both, or pretreatment and both. When preadipocytes are exposed to E0771 cancer conditioned medium during the induction phase and the cancer conditioned medium is removed for the duration of maintenance, there are no changes in gene expression profiles (Figure 28A). When preadipocytes undergo normal induction and then are exposed to cancer conditioned medium throughout the maintenance period, there is a significant *decrease* in mRNA expression of *Ucp1*, and decreases (though not statistically significant after correcting for multiple comparisons) in *Cidea* and *Ppargc1a* (peroxisome proliferative activated receptor, gamma, coactivator 1 alpha) (Figure 28B). When preadipocytes are exposed to cancer conditioned medium throughout both induction and maintenance phases of differentiation, statistically significant *decreases* in *Cidea*, *Ppargc1a*, and *Ucp1* are observed (Figure 28C). No changes are observed with pre-treatment before and during differentiation (Figure 28D). For all treatment groups, no changes in lipolysis-related genes *Lipe* or *Lpl* occurred.

We also asked if the time during maintenance was important. We demonstrate again that exposure to cancer conditioned medium before induction, or throughout induction, do not increase *Ucp1* expression (Figure 29A,B). Significant *decreases* in *Ucp1* mRNA expression were detected in mature adipocytes after exposure to cancer

conditioned medium at various timepoints throughout the maintenance period of differentiation (Figure 29C-F). Lastly, we demonstrate that exposure to cancer conditioned medium during both induction and maintenance results in the most dramatic and significant decrease in *Ucp1* and *Cidea* expression (Figure 29G).

While these results highlight the changes from cancer conditioned medium that can be observed in differentiating adipocytes, we next asked whether transdifferentiation could occur – whether *mature* white adipocytes could transdifferentiate into beige adipocytes – when exposed to cancer conditioned medium. This question is more physiologically relevant as there is only a small percentage of adipocyte turnover (preadipocyte differentiation into mature white adipocytes) *in vivo* [25]. We exposed fully mature white adipocytes to E0771 cancer conditioned medium exposure for 7 or 14 consecutive days. Interestingly, our results suggest that similar to differentiating adipocytes, mature white adipocyte exposure to cancer conditioned medium results in a significant *decrease* in *Ucp1* mRNA expression (Figure 30).

We next asked whether we would observe a similar decrease in *Ucp1* expression using cancer conditioned medium from another mouse cancer cell line. Using cancer conditioned medium from Lewis Lung Carcinoma (LLC) cells, we demonstrate that the effect of decreased *Ucp1* is consistent across exposure to cancer conditioned medium from these two distinct cell lines (Figure 31B). Lastly, we asked whether 24 hours of exposure could induce these changes. Our results suggest that 24 hours of exposure is sufficient to cause a decrease in *Ucp1* mRNA expression in our *in vitro* model with both cell lines (Figure 31C).

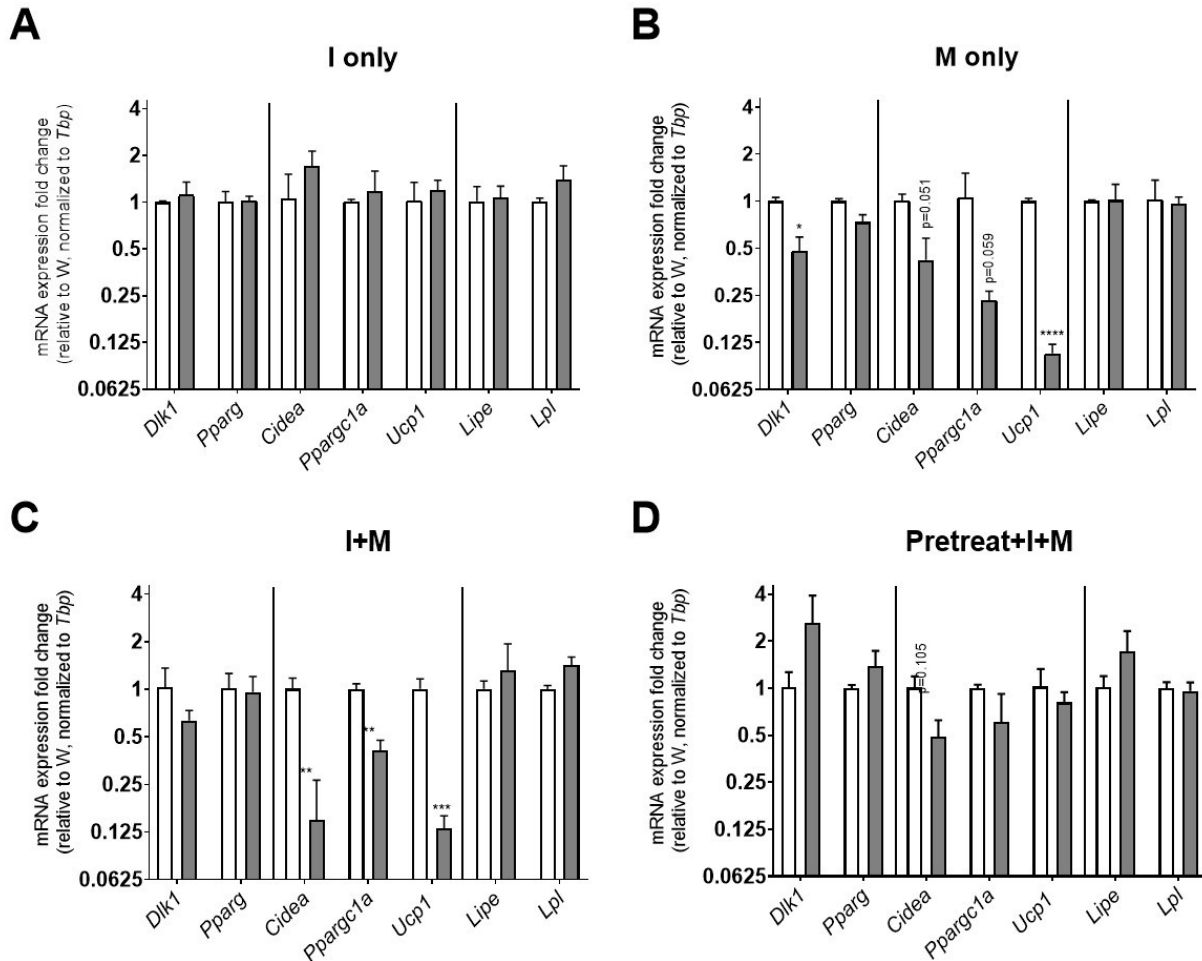


Figure 28. Mature adipocyte mRNA expression is altered after exposure to cancer conditioned medium during phases of differentiation; part 1

Immortalized SVF were exposed to either E0771 conditioned medium (gray bars) or control white medium (white bars) as described in methods. Quantitative PCR for a variety of adipogenesis and adipocyte gene markers in mature adipocytes after (A) cancer conditioned medium during only the induction phase ('I only') of differentiation, (B) cancer conditioned medium during only the maintenance phase ('M only') of differentiation, (C) cancer conditioned medium during both induction and maintenance phases ('I+M') of differentiation, and (D) cancer conditioned medium before differentiation and during both induction and maintenance phases ('Pretreat+I+M') of differentiation. Please note y-axis scale is log2. n = 3. Statistical tests: (A), (B), (C), and (D) Two-way ANOVA and Dunnett's multiple comparisons tests.

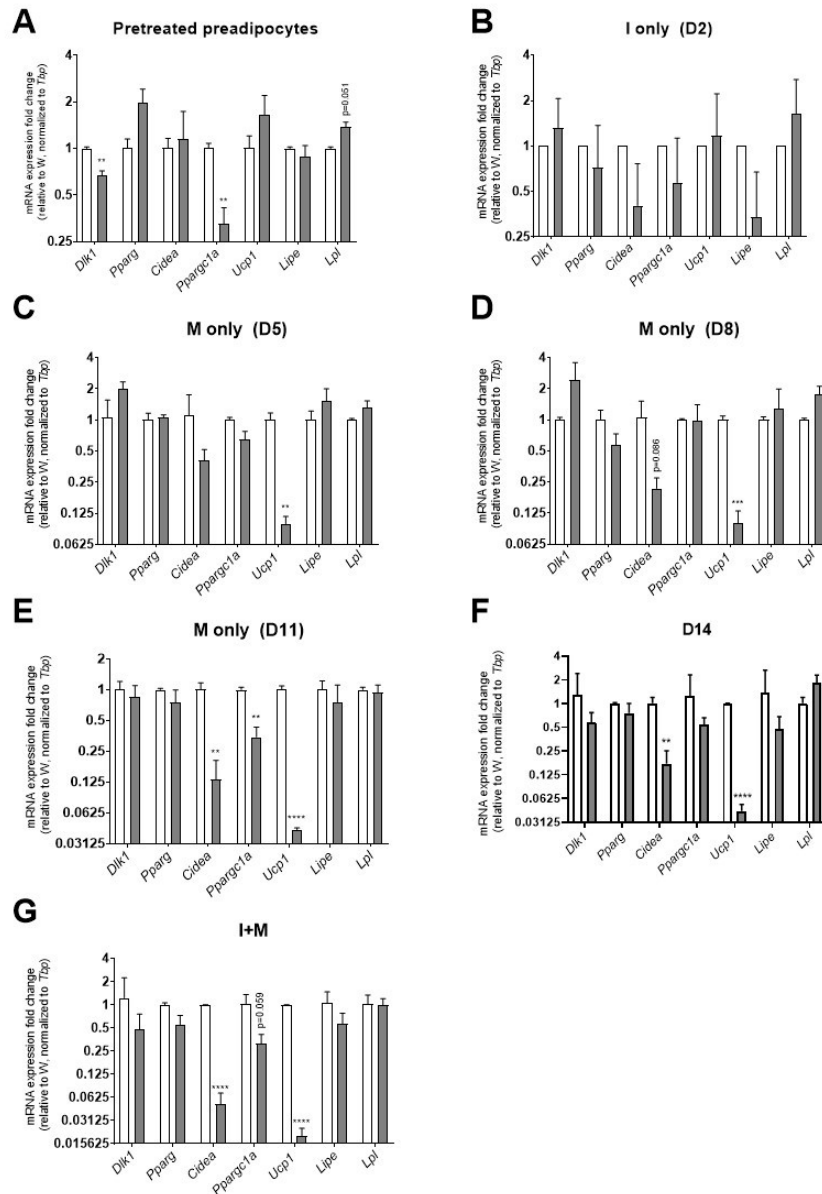


Figure 29. Mature adipocyte mRNA expression is altered after exposure to cancer conditioned medium during phases of differentiation; part 2

Immortalized SVF were exposed to either E0771 conditioned medium (gray bars) or control white medium (white bars) as described in methods. Quantitative PCR for a variety of adipogenesis and adipocyte gene markers in mature adipocytes after exposure to (A) cancer conditioned medium before differentiation ('pretreated preadipocytes'), (B) cancer conditioned medium during only the induction phase of differentiation ('I only (D2)'), (C) cancer conditioned medium during only the first three days of the maintenance phase ('M only (D5)'), (D) cancer conditioned medium during only the first six days of the maintenance phase ('M only (D8)'), (E) cancer conditioned medium during only the first nine days of the maintenance phase ('M only (D11)'), (F) cancer conditioned medium during only the entirety of the maintenance phase ('D14'), and (G) cancer conditioned medium during both induction and maintenance phases of differentiation ('I+M'). Please note y-axis scale is log2. n = 3. Statistical tests: (A), (B), (C), and (D) Two-way ANOVA and Dunnett's multiple comparisons tests.

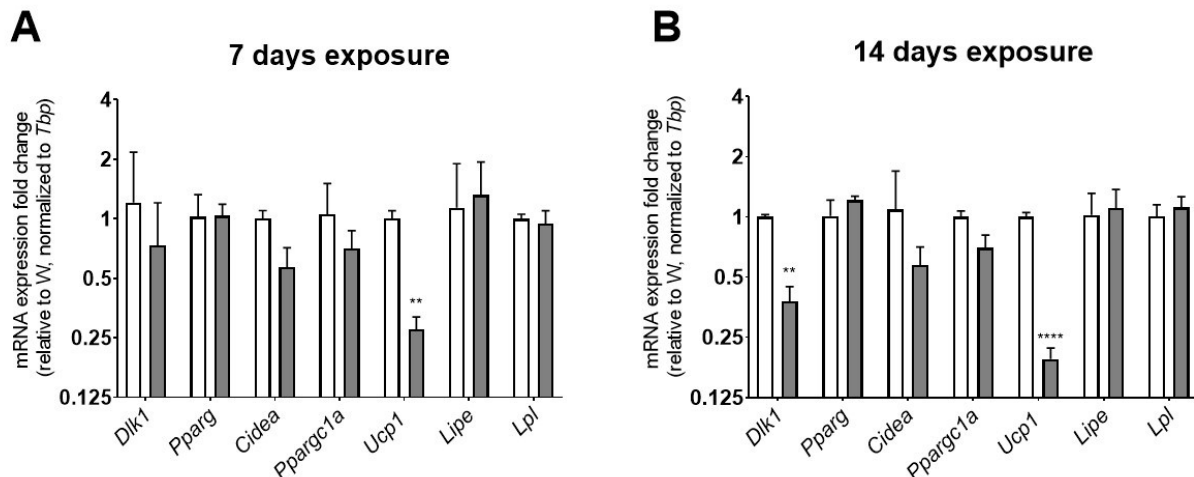


Figure 30. Mature adipocyte mRNA expression is altered after exposure to cancer conditioned medium after maturation

Immortalized SVF were exposed to either E0771 conditioned medium (gray bars) or control white medium (white bars) as described in methods. Quantitative PCR for a variety of adipogenesis and adipocyte gene markers in mature adipocytes after mature, fully differentiated white adipocytes were exposed to cancer conditioned medium for (A) 7 days or (B) 14 days. Please note y-axis scale is log2. n = 3. Statistical tests: (A), (B) Two-way ANOVA and Dunnett's multiple comparisons tests.

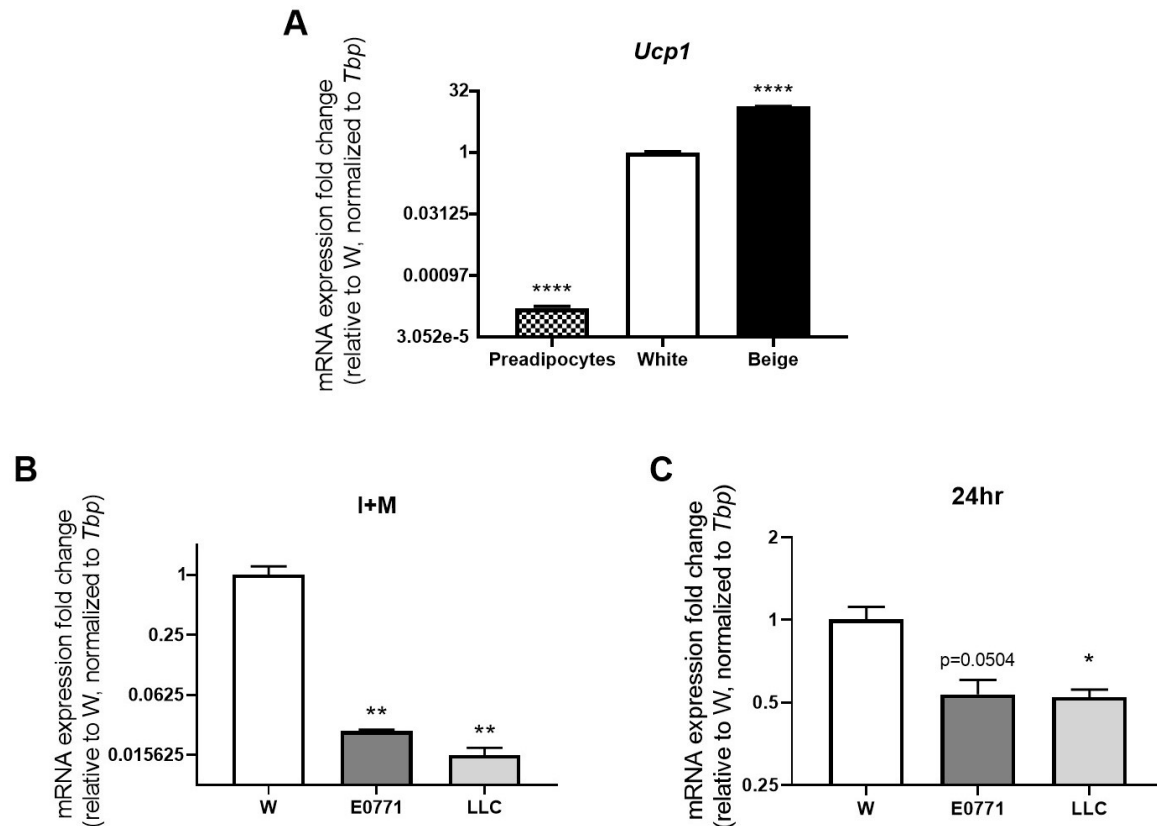


Figure 31. Mature adipocyte mRNA expression is altered after exposure to cancer conditioned medium both during and after maturation

(A) Quantitative PCR for *Ucp1* in immortalized SVF ('preadipocytes' in image) before differentiation and in mature white ('White' in image) and beige ('Beige' in image) adipocytes after differentiation. (B) and (C) Quantitative PCR for *Ucp1* in immortalized SVF exposed to cancer conditioned medium either (B) during differentiation or (C) after differentiation was completed as described in methods. For (B) and (C), white control is indicated as 'W,' 'E0771' represents E0771 conditioned medium, and 'LLC' represents LLC conditioned medium. Please note y-axis scale is log2. n = 3-5. Statistical tests: (A), (B), (C) Kruskal-Wallis and Dunn's multiple comparisons tests.

4.2.3 Primary murine stromal vascular fraction cells allow for *in vitro* differentiation to white and beige adipocytes.

Next, we established a *primary* cell culture system that would confer additional advantages to our methodical experiments to study the effects of breast cancer (and other cancers) on adipocytes *in vitro*. Primary SVF cells (also referred to as preadipocytes) are capable of undergoing differentiation to both mature white and beige adipocytes (Figure 32). Oil Red O staining demonstrates successful differentiation in both white and beige groups, with larger lipid droplets in the white differentiation group (Figure 32 and Figure 33A). Expression of white adipocyte-associated genes *Cfd* (complement factor D, also known as adipsin) and *Lep* (leptin) were significantly decreased in both preadipocytes (not expressed for leptin) and beige adipocytes relative to white adipocytes, and expression of *Fasn* (fatty-acid synthase) was significantly decreased in preadipocytes (Figure 33C). Lastly, expression of thermogenic adipocyte genes *Cidea*, *Ppargc1a*, *Tnfrsf9* (tumor necrosis factor receptor superfamily, member 9), and *Ucp1* were significantly increased in differentiated beige adipocytes (Figure 33D).

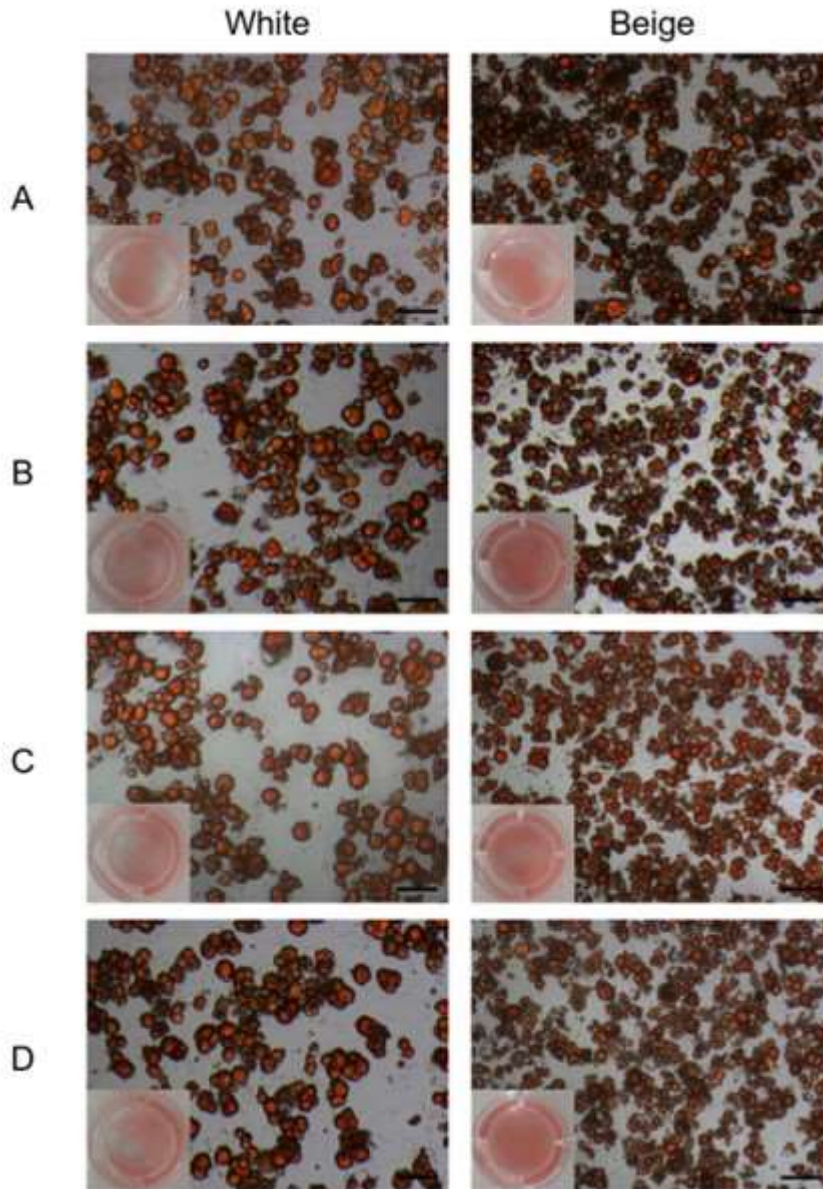


Figure 32. Primary murine inguinal SVF can differentiate into mature white and beige adipocytes

Oil Red O brightfield images from primary culture female inguinal white (left column) or beige (right column) adipocytes after full induction and maintenance periods (day 16). A,B,C,D represent 4 different female primary culture lines. Scale bars = 100 μ m.

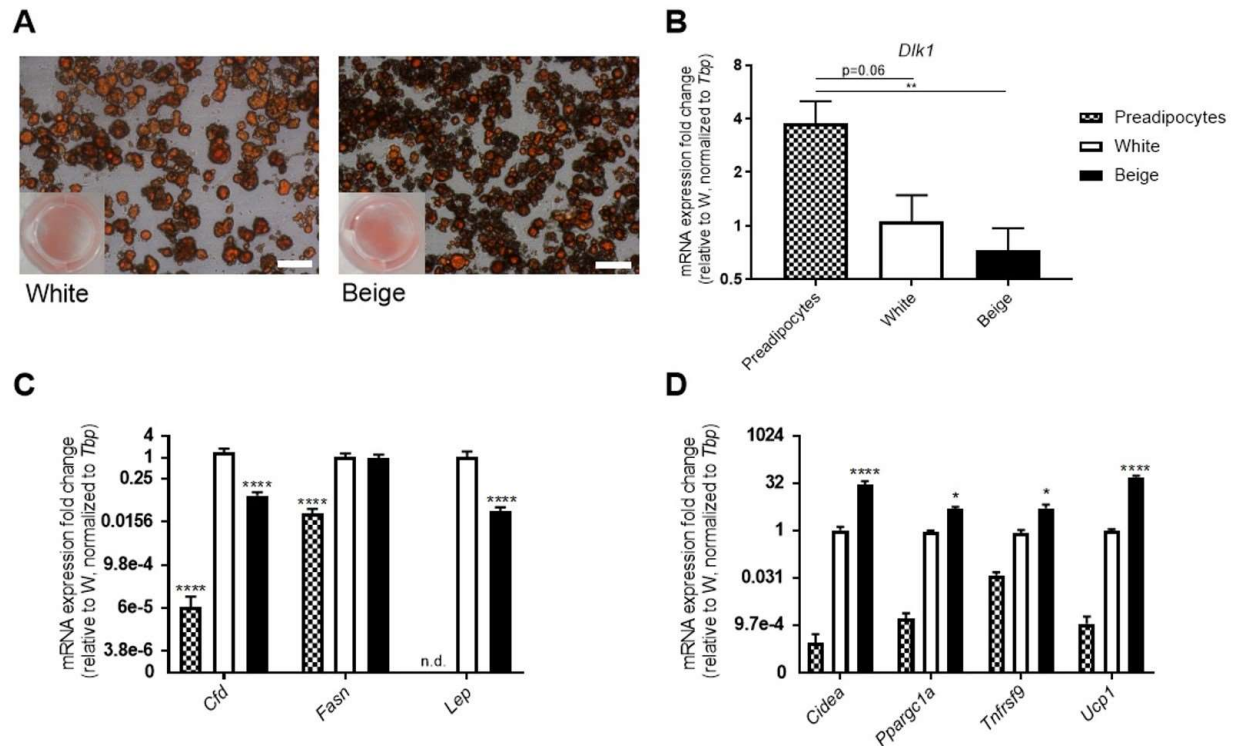


Figure 33. Primary murine SVF are capable of differentiation to mature white and beige adipocytes

Inguinal SVF isolated from young female C57BL/6J mice were differentiated to either white or beige adipocytes as described in methods. (A) Representative Oil Red O staining confirming that our protocol is highly selective for culture of preadipocytes capable of undergoing successful differentiation. Scale bars = 100 μ m. Quantitative PCR for (B) *Dlk1*, (C) markers of white adipocytes, (D) markers of beige adipocytes. Please note y-axis scale is log2. $n = 5$ primary SVF cell lines. Error bars = standard deviation. Statistical tests: (B) Kruskal-Wallis and Dunn's multiple comparisons tests, (C) and (D) Two-way ANOVA and Dunnett's multiple comparisons tests. SVF = stromal vascular fraction, n.d. = not detected.

4.2.4 Cancer conditioned medium and coculture alter white adipocyte gene expression, but do not increase thermogenic gene expression in murine primary differentiated white adipocytes

To further investigate the localized WAT browning observed *in vivo* in Chapter 3, we exposed differentiated, mature white adipocytes from primary mouse SVF to the conditioned medium from E0771 murine breast cancer cells (Figure 34), or cocultured them with E0771 cells (Figure 36). *Ucp1* mRNA expression in white adipocytes exposed to E0771 cancer-conditioned medium was decreased, although not significantly, relative to control white adipocytes, and was significantly decreased when compared to both control beige adipocytes and white adipocytes exposed to the $\beta 3$ adrenergic agonist CL 316,243 (Figure 34A). Western blot of UCP1 confirms these mRNA expression results on the protein level (Figure 35).

Cancer-conditioned medium exposure did not result in increased expression of any of the thermogenic genes tested, except a trend towards higher *Tnfrsf9* (Figure 34B). Interestingly, a significant decrease in the white adipocyte-associated genes *Fasn* and *Lep* was observed in the cancer-conditioned medium treated adipocytes (Figure 34C). We also tested for genes associated with lipolysis, and found that white adipocytes exposed to E0771 cancer-conditioned medium had no changes in expression of *Lpl* or *Mgll* (monoglyceride lipase), but expressed less *Lipe* compared to control white adipocytes (Figure 34D).

When differentiated white adipocytes were cocultured with E0771 cells, the same pattern of *Ucp1* expression was observed: a decrease (although not statistically significant) in *Ucp1* expression compared to control white adipocytes, but significantly

lower *Ucp1* expression compared to white adipocytes treated with CL 316,243 (Figure 36A). While there were no changes in gene expression of the thermogenic genes *Cidea* or *Ppargc1a*, white adipocytes cocultured with E0771 cells expressed significantly more *Tnfrsf9* (Figure 36B). Similar to the cancer-conditioned medium results (Figure 34), white adipocytes cocultured with E0771 cells expressed significantly less *Fasn* and *Lep* mRNA compared to white control adipocytes, with no change in *Cfd* expression (Figure 36C). Lastly, expression of *Mgll* was significantly increased in white adipocytes cocultured with E0771 cells, while expression of *Lipe* and *Lpl* was decreased but not significantly (Figure 36D).

Lastly, we asked if these results could be replicated using a different murine mouse model for SVF and breast cancer (BALB/c mice and the 4T1 breast cancer cell line). We show, similar to Figure 34 and Figure 35, that exposure of mature white adipocytes from BALB/c mice to 4T1 cancer conditioned medium decreases *Ucp1* mRNA expression (Figure 37A) and UCP1 protein expression (Figure 38). 4T1 cancer conditioned medium exposure resulted in only a 2-fold increase in *Tnfrsf9*, which was not statistically significant (Figure 37B). Of the white adipocyte-associated genes tested, only *Lep* expression was significantly decreased with 4T1 exposure (Figure 37C). Lastly, although none of the lipolysis-related genes were significantly different after 4T1 cancer conditioned medium exposure, there were slight trends of decreasing *Lpl* expression and increasing *Mgll* expression (Figure 37D).

Coculture of BALB/c -derived mature white adipocytes with 4T1 cells resulted in relatively similar gene expression patterns (Figure 39): no change in *Ucp1* expression (Figure 39A), non-statistically-significant 4-fold increases in *Tnfrsf9* expression (Figure

39B) and 2-fold decreases in *Lep* expression (Figure 39C), and no changes in lipolysis-related gene expression (Figure 39D).

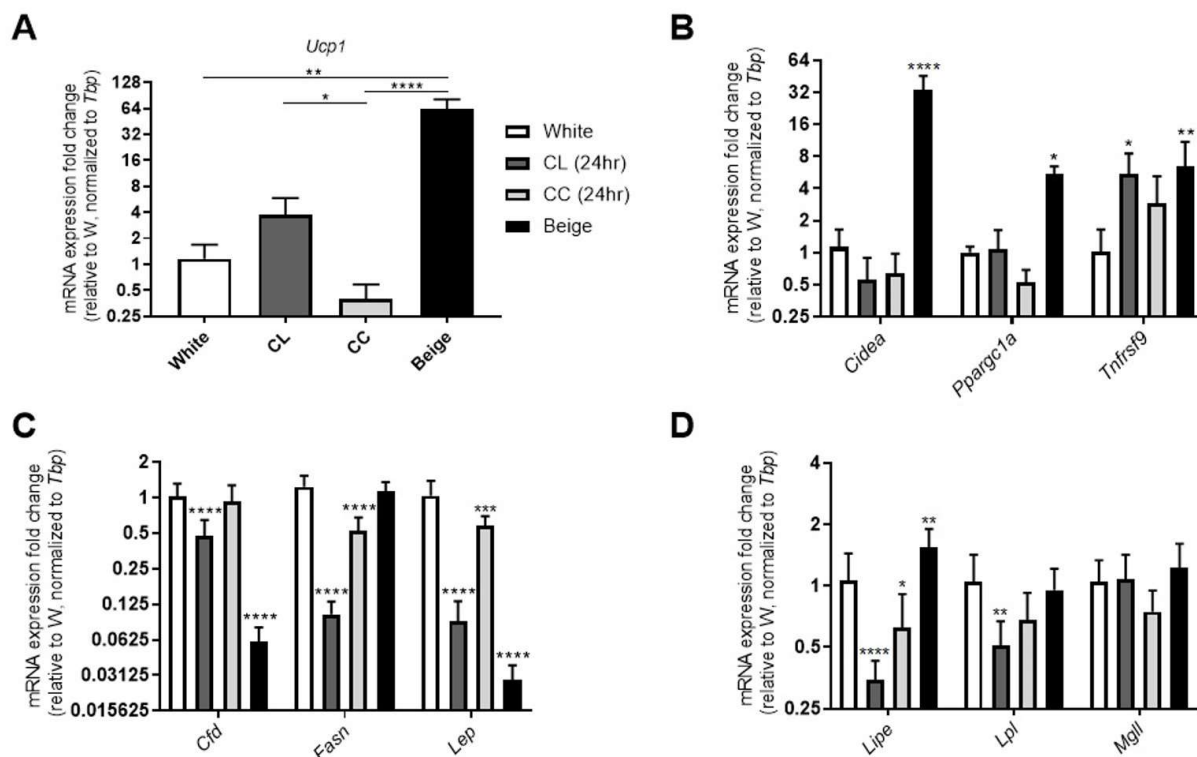


Figure 34. E0771 cancer-conditioned medium alters white adipocyte mRNA expression, but does not induce white adipocyte browning in vitro

Differentiated white adipocytes were treated with CL 316,243 (noted as 'CL' in images) or E0771 cancer-conditioned medium (noted as 'CC' in images) and compared to controls as described in methods. Quantitative PCR for (A) *Ucp1*, (B) other markers of beige adipocytes, (C) markers of white adipocytes, and (D) markers of lipolysis. Please note y-axis scale is log2. n = 7 primary C57BL/6J SVF cell lines. Statistical tests: (A) Kruskal-Wallis and Dunn's multiple comparisons tests, (B), (C), and (D) Two-way ANOVA and Dunnett's multiple comparisons tests.

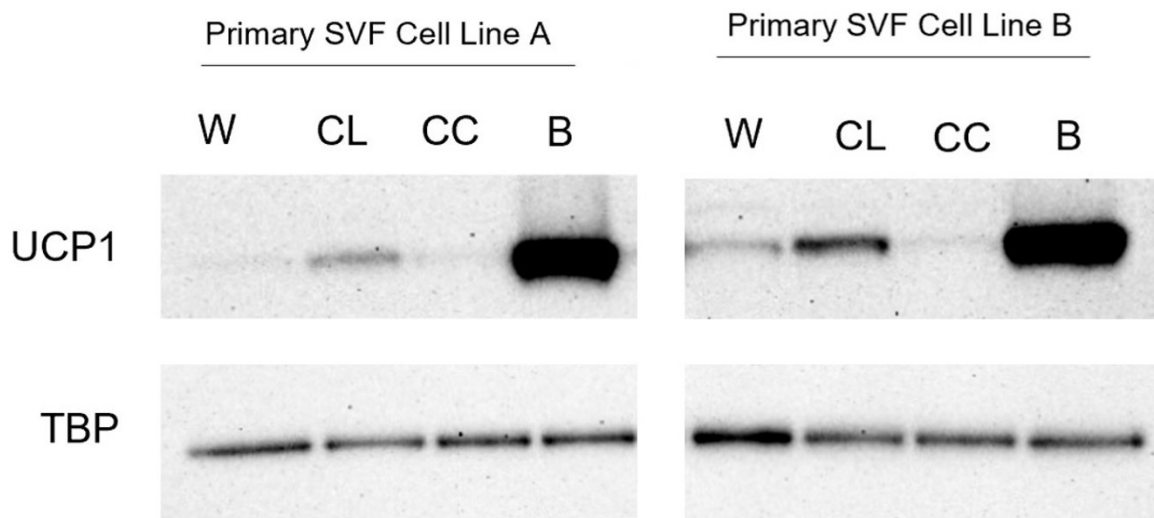


Figure 35. Representative western blot of UCP1 and TBP demonstrating that E0771 cancer-conditioned medium does not induce white adipocyte browning in vitro

Differentiated white adipocytes were treated with CL 316,243 (noted as 'CL' in images) or E0771 cancer-conditioned medium (noted as 'CC' in images) and compared to white 'W' and beige 'B' controls. Western blot performed in two of the seven primary C57BL/6J SVF cell lines used.

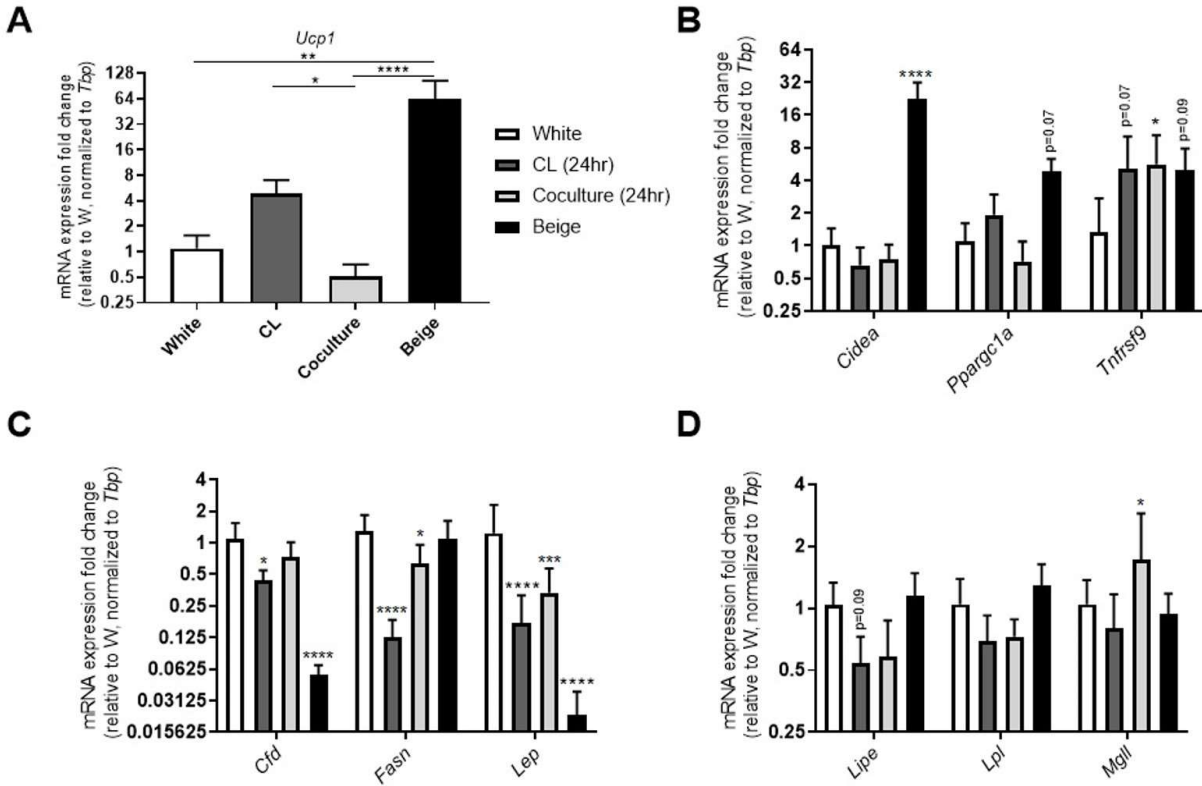


Figure 36. E0771 coculture alters white adipocyte mRNA expression, but does not induce white adipocyte browning in vitro.

Differentiated white adipocytes were treated with CL 316,243 (noted as 'CL' in images) or cocultured with E0771 cells (noted as 'Coculture' in images) and compared to controls as described in methods. Quantitative PCR for (A) *Ucp1*, (B) other markers of beige adipocytes, (C) markers of white adipocytes, and (D) markers of lipolysis. Please note y-axis scale is log2. n = 7 primary C57BL/6J SVF cell lines. Statistical tests: (A) Kruskal-Wallis and Dunn's multiple comparisons tests, (B), (C), and (D) Two-way ANOVA and Dunnett's multiple comparisons tests.

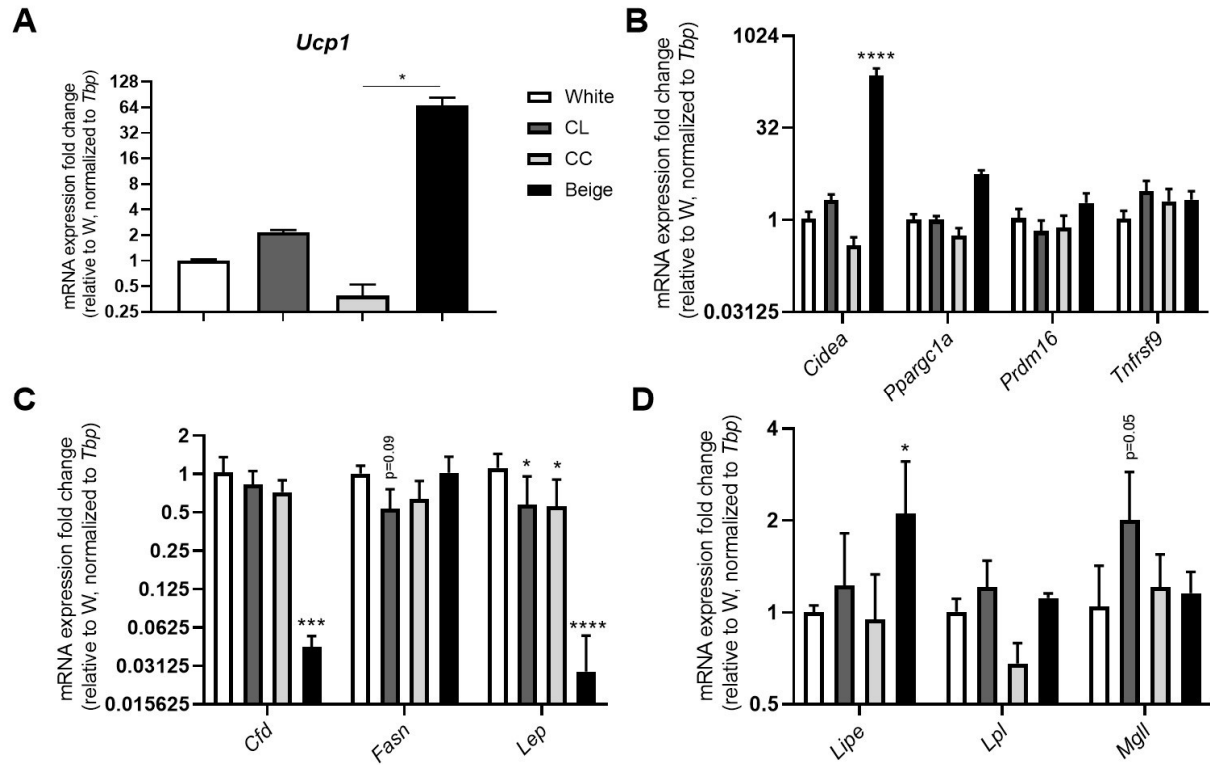


Figure 37. 4T1 cancer conditioned medium alters white adipocyte mRNA expression, but does not induce white adipocyte browning in vitro

Differentiated white adipocytes were treated with CL 316,243 (noted as 'CL' in images) or 4T1 cancer-conditioned medium (noted as 'CC' in images) and compared to controls as described in methods. Quantitative PCR for (A) *Ucp1*, (B) other markers of beige adipocytes, (C) markers of white adipocytes, and (D) markers of lipolysis. Please note y-axis scale is log2. n = 3 primary BALB/cJ SVF cell lines. Statistical tests: (A) Kruskal-Wallis and Dunn's multiple comparisons tests, (B), (C), and (D) Two-way ANOVA and Dunnett's multiple comparisons tests.

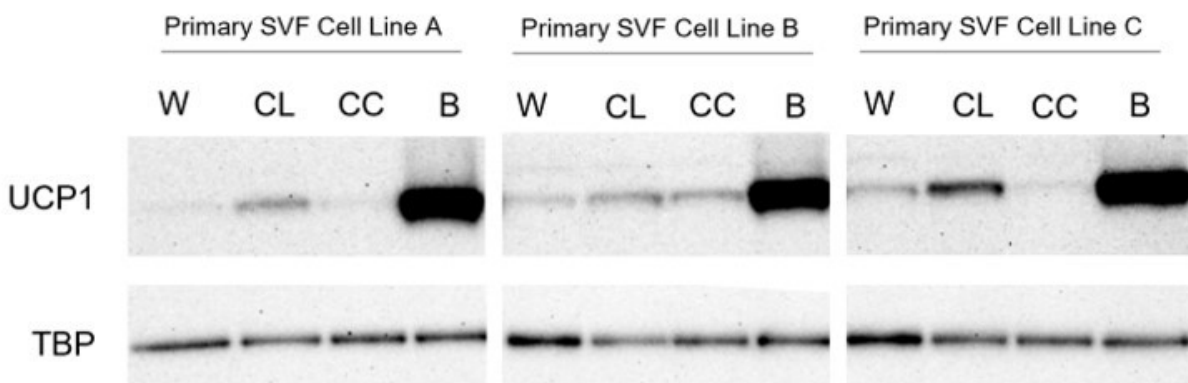


Figure 38. Western blot of UCP1 and TBP demonstrating that 4T1 cancer-conditioned medium does not induce white adipocyte browning in vitro

Differentiated white adipocytes were treated with CL 316,243 (noted as 'CL' in images) or 4T1 cancer-conditioned medium (noted as 'CC' in images) and compared to white 'W' and beige 'B' controls. Western blot performed in all three of the BALB/cJ SVF cell lines used.

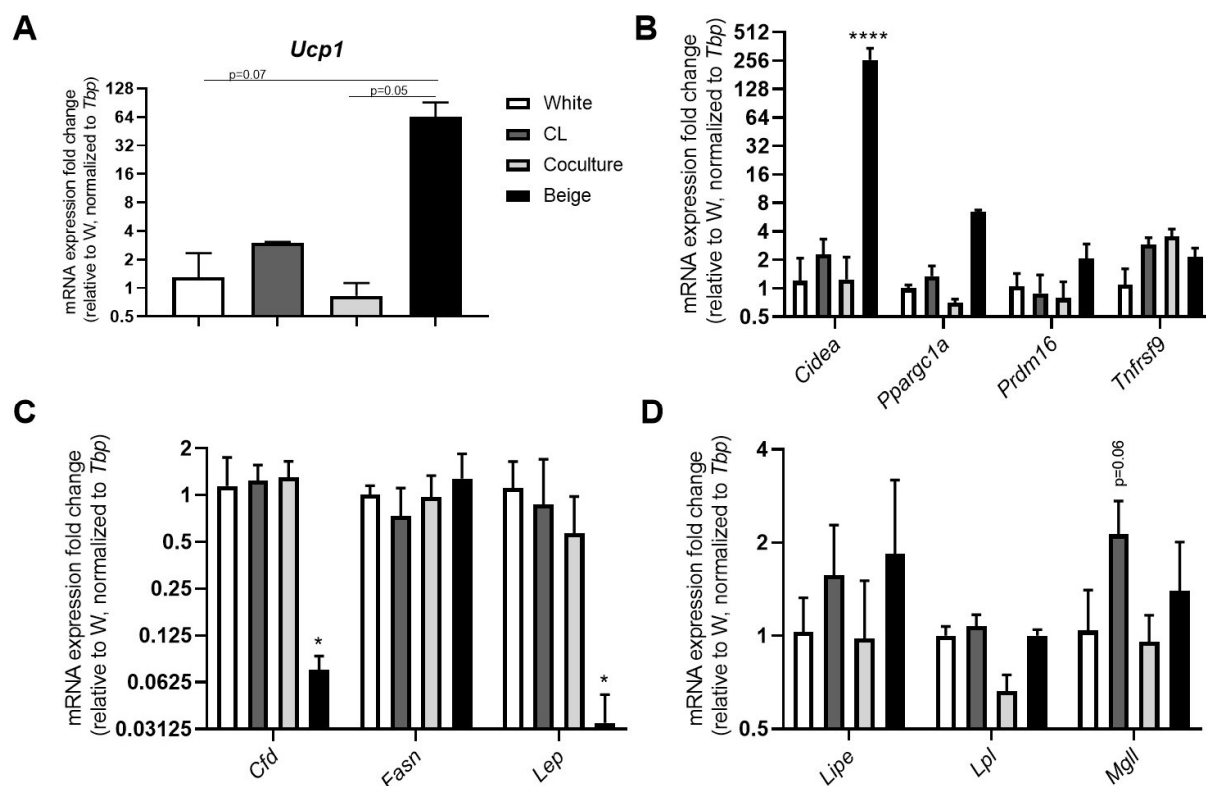


Figure 39. 4T1 coculture alters white adipocyte mRNA expression, but does not induce white adipocyte browning in vitro.

Differentiated white adipocytes were treated with CL 316,243 (noted as 'CL' in images) or cocultured with 4T1 cells (noted as 'Coculture' in images) and compared to controls as described in methods. Quantitative PCR for (A) *Ucp1*, (B) other markers of beige adipocytes, (C) markers of white adipocytes, and (D) markers of lipolysis. Please note y-axis scale is log2. n = 3 primary BALB/cJ SVF cell lines. Statistical tests: (A) Kruskal-Wallis and Dunn's multiple comparisons tests, (B), (C), and (D) Two-way ANOVA and Dunnett's multiple comparisons tests.

4.2.5 Human breast-derived stromal vascular fraction cells allow for in vitro differentiation to white and beige adipocytes

We wanted to determine whether changes that occurred in murine cell culture experiments could be replicated with human cell culture. Based on the results in mouse cell lines, we decided to use human SVF. We tested differentiation requirements in female human breast SVF and found that they require additional supplementation in their cell culture medium in order to successfully differentiate, as demonstrated by differences in Oil Red O staining (Figure 40).

We demonstrate that our human SVF cell culture system, similar to in mice, is capable of differentiating into mature white and beige adipocytes with adequate differentiation and larger lipid droplet accumulation in mature white adipocytes (Figure 41A). Additionally, *UCP1* mRNA expression is significantly increased in our beige adipocytes compared to white, and was not detected at all in the undifferentiated SVF/preadipocytes (Figure 41B). These results demonstrate a viable human *in vitro* model for studying the effects of tumor and tumor microenvironment on adipocyte plasticity.

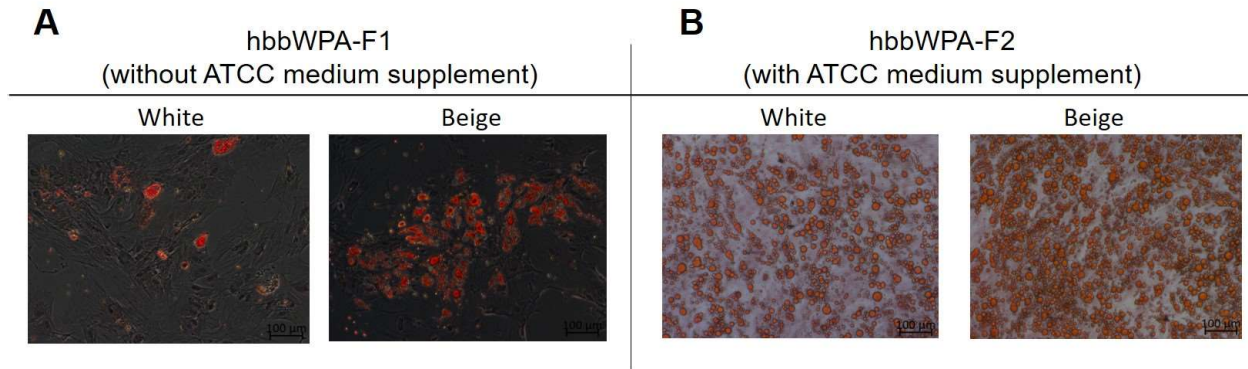


Figure 40. Human breast SVF requires additional medium supplementation to allow for robust differentiation to mature white and beige adipocytes.

Isolated white preadipocytes from female normal breast tissue obtained during breast reduction surgery were differentiated to mature white and beige adipocytes with or without supplementation with the ATCC Low Serum Fibroblast Growth Kit as described in methods. Representative Oil Red O images demonstrate (A) poor differentiation percentage in cells without supplementation, (B) robust differentiation in cells with supplementation. Scale bars = 100µm. hbbWPA = human benign breast white preadipocytes.

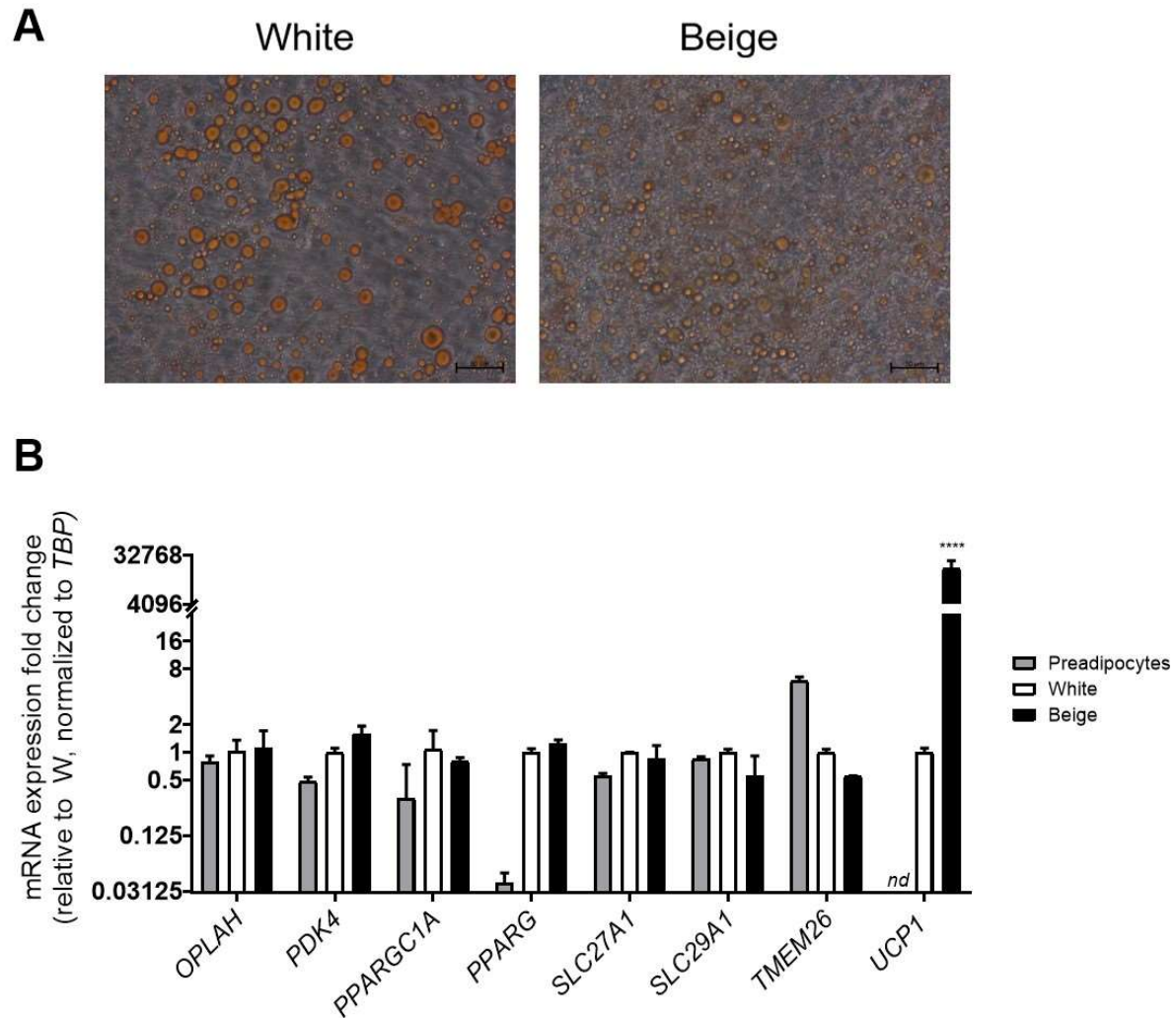


Figure 41. Primary human SVF are capable of differentiation to mature white and beige adipocytes.

SVF cells isolated from human female normal WAT were differentiated to either white or beige adipocytes as described in methods. (A) Representative Oil Red O staining confirming that our protocol is highly selective for culture of preadipocytes capable of undergoing successful differentiation. Scale bars = 50µm. (B) Quantitative PCR for thermogenic adipose tissue-related genes. N = 2. Statistical tests for (B) Two-way ANOVA and Dunnett's multiple comparisons tests. SVF = stromal vascular fraction, n.d. = not detected.

4.2.6 Cancer conditioned medium induces changes to white adipocyte gene expression in human primary differentiated white adipocytes

The first question using our human SVF cell culture system, similar to that in our murine SVF cell culture system, was whether exposure to breast cancer conditioned medium at various points in the differentiation process would result in WAT browning. As described in methods section 2.8.3 (Figure 9), human SVF cells were exposed to MCF-7 and MDA-MB-231 cancer conditioned medium at various stages of differentiation: either the entire induction phase, partial or entire maintenance phase, or both induction & maintenance periods. Compared to mature white adipocytes, exposure to MCF-7 or MDA-MB-231 cancer conditioned medium during only the induction phase of differentiation resulted in no changes in *Ucp1*, but significantly decreased *TMEM26* (transmembrane protein 26) expression (Figure 42A). Expression of several other thermogenic adipose tissue-related genes such as *EAR2* (nuclear receptor subfamily 2 group F member 6), *OPLAH*, and *PPARGC1A* were decreased, while *PDK4* (mitochondrial pyruvate dehydrogenase (acetyl-transferring) kinase isozyme 4) expression was approximately 2-fold higher in both cancer conditioned medium treated groups, but no changes were significant (Figure 42A). Exposure to MCF-7 or MDA-MB-231 cancer conditioned medium during only the first half of the maintenance phase of differentiation resulted in significant decreases in many of the genes tested, although there was no change in *UCP1* expression (Figure 42B). When differentiating white adipocytes were exposed to MCF-7 or MDA-MB-231 cancer conditioned medium throughout the entirety of the maintenance phase of differentiation, there were still trends towards decreased expression of many of the thermogenic adipose tissue-

related genes; however, for *UCP1* expression, there was a 2.2-fold increase (not statistically significant) in the group treated with MCF-7 conditioned medium, and a statistically significant 3.8-fold increase in the group treated with MDA-MB-231 conditioned medium (Figure 42C). When exposure to cancer conditioned medium spanned the entirety of the induction and maintenance phases, the MDA-MB-231 conditioned medium treatment group expressed significantly more *UCP1*, roughly 6.4-fold more than mature white adipocyte controls (Figure 42D).

We also analyzed the Oil Red O images and quantified lipid droplet sizes. Control beige adipocytes have significantly smaller lipid droplets than mature white adipocytes (Figure 43A). Regardless of the timing or duration of cancer conditioned medium exposure, maturing white adipocytes exposed to either MCF-7 or MDA-MB-231 cancer conditioned medium had significantly smaller lipid droplets when compared to white adipocyte controls (Figure 43B-E).

Lastly, we tested the effects of these cancer conditioned mediums on mature white adipocytes in an SVF cell line derived from a different human patient during differentiation and after maturation for 24 hours. Interestingly, although this SVF cell line had appropriate *UCP1* expression levels in undifferentiated, mature white, and mature beige adipocytes (Figure 44A), exposure to MCF-7 and MDA-MB-231 cancer conditioned medium throughout differentiation resulted in a significant decrease in *UCP1* expression (Figure 44B). Exposure to mature white adipocytes to cancer conditioned medium for 24 hours resulted in slightly different results, with a roughly 2-fold increase in *UCP1* expression in the MCF-7 conditioned medium group, and 8-fold

decrease in *UCP1* expression in the MDA-MB-231 conditioned medium group (Figure 44C).

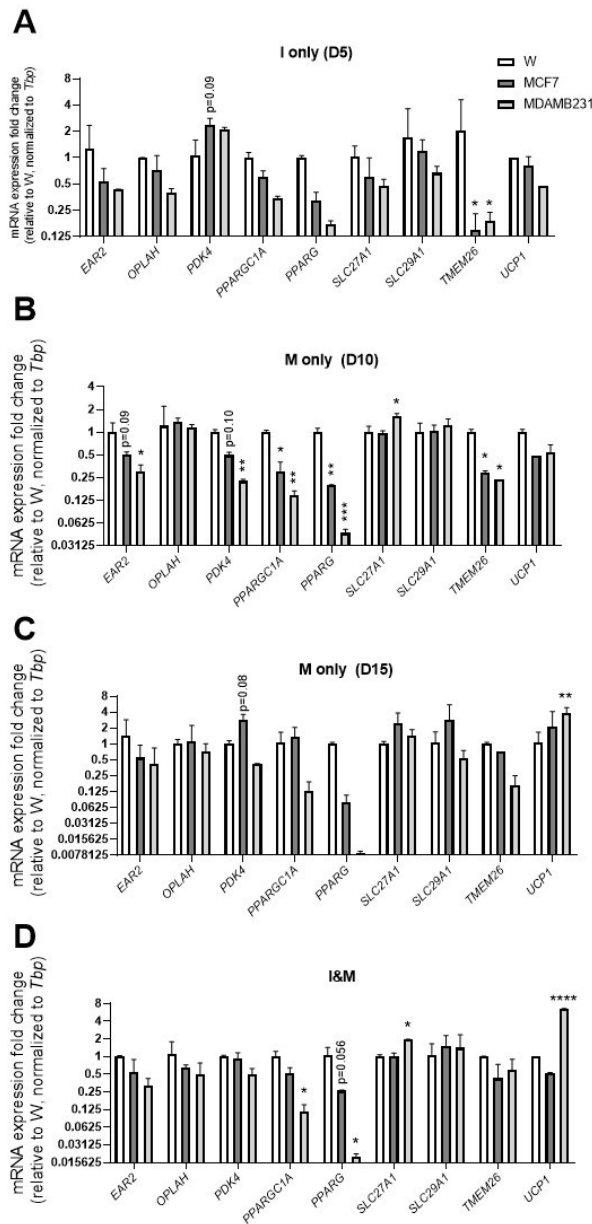


Figure 42. Mature human adipocyte mRNA expression is differentially altered after exposure to MCF-7 and MDA-MB-231 cancer conditioned mediums during phases of differentiation

Primary SVF derived from normal WAT from a 27 year old woman were exposed to either MCF-7 conditioned medium (dark gray bars), MDA-MB-231 conditioned medium (light gray bars), or control white medium (white bars) as described in methods. Quantitative PCR for a variety of adipogenesis and adipocyte gene markers in mature adipocytes after exposure to (A) cancer conditioned medium during only the induction phase of differentiation ('I only (D5)'), (B) cancer conditioned medium during only the first half of the maintenance phase ('M only (D10)'), (C) cancer conditioned medium during only the entirety of the maintenance phase ('M only (D15)'), and (D) cancer conditioned medium during both induction and maintenance phases of differentiation ('I&M'). Please note y-axis scale is log2. n = 2-3. Statistical tests: (A), (B), (C), and (D) Two-way ANOVA and Dunnett's multiple comparisons tests.

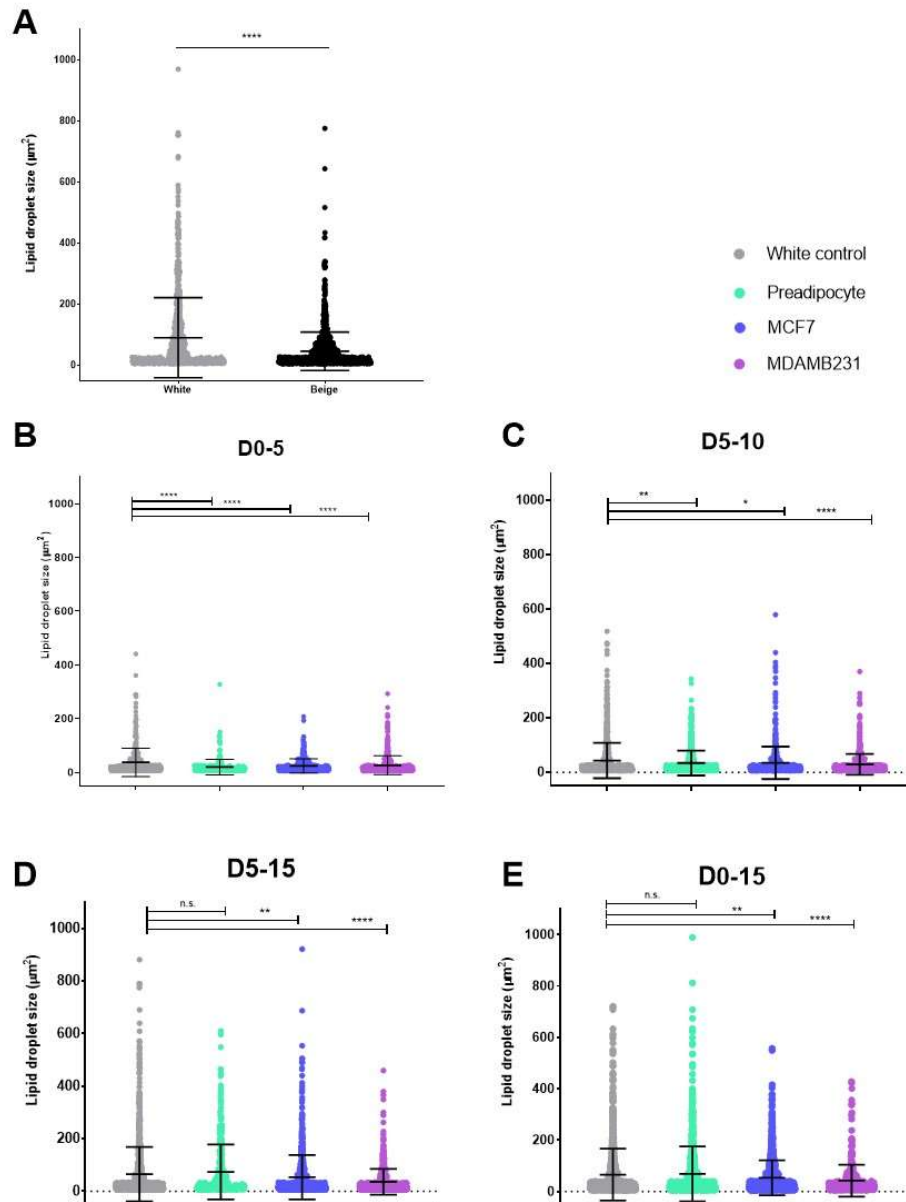


Figure 43. Lipid droplet size significantly decreases in mature human adipocytes after exposure to MCF-7 and MDA-MB-231 cancer conditioned mediums during differentiation

Primary SVF derived from benign breast WAT from a 27 year old woman were differentiated to mature white or beige adipocytes. Lipid droplet sizes in (A) white (gray dots) versus beige (black dots) control adipocytes, (B) cancer conditioned medium during only the induction phase of differentiation, (C) cancer conditioned medium during only the first half of the maintenance phase, (D) cancer conditioned medium during only the entirety of the maintenance phase, and (E) cancer conditioned medium during both induction and maintenance phases of differentiation. For (B), (C), (D), and (E), MCF-7 conditioned medium (blue dots), MDA-MB-231 conditioned medium (magenta dots), or control white medium (gray dots) N=2 wells per group, with over 300 lipid droplets analyzed per condition. Statistical tests: (A) Unpaired t-test without assuming same standard deviation between groups; (B), (C), (D), (E) One-way ANOVA and Dunnett's multiple comparisons tests.

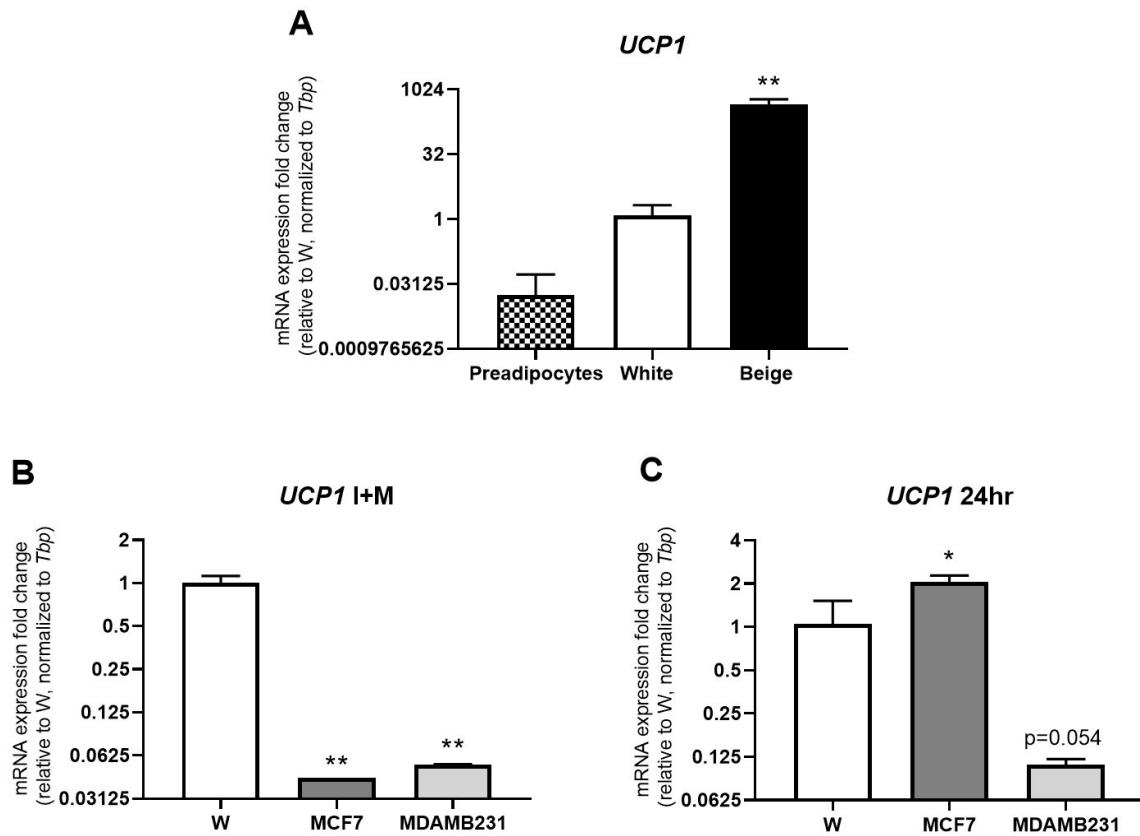


Figure 44. Mature adipocyte mRNA expression is differentially altered after exposure to MCF-7 and MDA-MB-231 cancer conditioned mediums both during and after maturation

(A) Quantitative PCR for *Ucp1* in primary human SVF derived from normal WAT from a 51 year old woman ('preadipocytes' in image) before differentiation and in mature white ('White' in image) and beige ('Beige' in image) adipocytes after differentiation. (B) and (C) Quantitative PCR for *UCP1* in primary human SVF exposed to cancer conditioned medium either (B) during differentiation or (C) after differentiation was completed as described in methods. For (B) and (C), white control is indicated as 'W,' 'MCF7' represents MCF-7 conditioned medium, and 'MDAMB231' represents MDA-MB-231 conditioned medium. Please note y-axis scale is log₂. n = 2-3. Statistical tests: (A), (B), (C) Kruskal-Wallis and Dunn's multiple comparisons tests.

4.3 Discussion

We utilized our lab's immortalized white SVF, derived from the inguinal WAT of a male C57BL/6J mouse, to produce an *in vitro* model of adipocyte differentiation that could be used to investigate the role of the cancer cell secretome in adipocyte plasticity both during and after adipocyte differentiation. E0771, an estrogen-receptor positive murine breast cancer cell line, is commonly used in many *in vitro* and *in vivo* C57BL/6 breast cancer studies [167-170]. Use of an established breast cancer cell line, as opposed to the use of whole tumor lysate, provides a strategic advantage in the mechanistic study of WAT browning *in vitro* because it is cultured without other cells within the tumor microenvironment, which could confound results, and eliminates the problems of inter- and intra-organism tumor heterogeneity [165, 166]. However, these are not perfect replacements for cancer cells isolated from tumors grown *in vivo*, and this should be kept in mind when interpreting our experimental results.

Our results using cancer conditioned medium from E0771 cells suggest that exposure to cancer conditioned medium during the initial induction phase of differentiation does not impact mature adipocyte gene expression; instead, exposure during the induction *and* maintenance phase or during the maintenance phase alone appears to have the most significant effects (Figure 28, Figure 29). This time-specificity is not surprising, as other studies have influenced adipogenesis at time-specific periods of differentiation. In 3T3-L1 'white preadipocytes,' mature adipocytes can initiate induction of differentiation of nearby preadipocytes, suggesting a role of endogenous paracrine signaling in the induction phase [171]. Also in 3T3-L1 preadipocytes, adipogenesis can be thwarted by retinoic acid-mediated decreases in PPARgamma and

C/EBPalpha, but only when exposed during the induction phase [172, 173]. Even in differentiation of brown adipocytes, STAT3 is required during the induction phase, but not the maintenance phase [174]. Other factors, such as Lipin 1 (an enzyme involved in lipid biosynthesis) and Microprocessor complex subunit DGCR8 (a protein critical for miRNA biogenesis), have been shown to be necessary for both differentiation *and* maintenance of white and/or brown adipocytes [175, 176]. Our results suggest that exposure during the initiation period must be maintained throughout maintenance, or even introduced just during the maintenance phase alone, in order to alter adipocyte mRNA expression in the context of cancer

We were surprised to observe that cancer conditioned medium exposure results in a *decrease* in mRNA expression of several thermogenic gene markers – *Ucp1*, *Cidea*, and *Ppargc1a* – opposite our initial hypothesis. This suggests, contrary to other studies, that the cancer cell secretome is *not* a direct driver of WAT browning *in vitro*. Additionally, no changes in lipolysis-related gene expression via *Lipe* or *Lpl* were identified in any of the treatment groups, further negating our hypothesized *direct* role of cancer conditioned medium on adipocyte changes in this model.

While these results give valuable insight into the effects of cancer conditioned medium on *differentiating* white preadipocytes, adipocyte turnover *in vivo* is quite low, with an annual turnover rate measured at approximately 10% in adults [25]. This means that the majority of cells impacted by any perturbations to surrounding environment are mature, rather than differentiating, adipocytes. We therefore tested whether E0771 cancer conditioned medium would induce transdifferentiation of white to beige adipocytes. We demonstrate that exposure of mature, fully differentiated, white

adipocytes to the conditioned medium of E0771 cells does *not* induce WAT browning, but instead results in a *decrease* in *Ucp1* expression. Again, these results were opposite our initial hypothesis. Furthermore, this decrease in *Ucp1* expression occurred regardless of whether the exposure lasted for one week, two weeks, or even just 24 hours (Figures 27, 28).

This prompted the use of 24 hour exposure periods for subsequent experiments discussed later in this discussion, as this shorter timeframe provides several advantages: it allows us to study more acute signaling changes which may initiate the long term changes; it decreases the need for multiple medium changes which introduces inherent variability (although we do our best to ensure consistency between each harvest of cancer conditioned medium); it decreases risk of adipocyte detachment before harvesting (based on our lab's observations, the risk for detachment increases the longer the mature adipocytes remain in culture, even with the use of gelatin-coated cell culture plates).

Lastly, we demonstrate that decreased *Ucp1* expression is not a response limited to the E0771 cell line. A similar, and to an extent more robust, decrease in *Ucp1* mRNA expression occurred after exposure to LLC conditioned medium (Figure 31). LLC cancer conditioned medium has been shown to induce muscle catabolism contributing to CAC [177], but more importantly has been implicated in PTHrP-mediated WAT browning in LLC tumor-driven cachexia in mice [178]. While we did not test our conditioned medium for the presence of PTHrP, we used a filter with pore sizes that would allow passage of PTHrP into the filtered medium used for adipocyte treatment, so our results suggest that any PTHrP-mediated changes are *not* direct in nature. Obtaining the same results – a

decrease in *Ucp1* mRNA expression – with two completely different cancer cell lines adds significant confidence to our conclusion that the cancer cell secretome is not the driver of WAT browning in this cell culture model.

However, several key limitations to this model exist. These SVF cells, similar to the commercially-available 3T3-L1 preadipocyte cell line [179], are derived from males, not female mice. It is well-established that metabolic differences exist between sexes in both mice and humans [180-182]. Use of a female SVF cell line would be advantageous for breast cancer studies. These male SVF cells were immortalized with SV40 Large T antigen, which inherently alters the genetic composition of these cells [160]. Although we demonstrate that these cells are capable of differentiating into white and beige adipocytes based on mRNA expression data (Figure 27) and have used these cells for other adipogenesis-related studies [183], we cannot be certain that the decrease in *Ucp1* expression is confounded by the immortalized nature of these cells. While cell lines offer several advantages, they are not always true replacements of primary cells [184]; studies comparing other primary versus immortalized cells have identified differential protein expression patterns that may lead to different experimental results [185]. Lastly, these studies were performed with experimental replicates using the same male immortalized SVF cell line, as opposed to SVF cells derived from different individual mice. With these three limitations in mind, we sought to establish a more robust and reliable cell culture system to test whether cancer conditioned medium could cause WAT browning.

We have established a viable cell culture system using primary inguinal SVF from female mice (Figure 32, Figure 33). The mRNA expression profiles of the

preadipocytes, differentiated white adipocytes, and differentiated beige adipocytes are appropriate for each respective cell type when compared to those of other models and whole tissue lysates [186]. Qualitatively, there appears to be a more noticeable difference between the lipid droplet sizes in mature white versus beige adipocytes in these primary cell lines (Figure 33A) when compared to the immortalized cell line (Figure 27A), suggesting that the immortalized preadipocyte cell line may in fact produce a difference in baseline differentiation. Quantitatively, there were more significant differences in mRNA expression values between preadipocytes, white adipocytes, and beige adipose when using these primary SVF (Figure 33 versus the immortalized SVF Figure 27). These significant differences are even more robust since they come from the consolidation of data from adipocytes differentiated from SVF derived from five *different, individual* female mice.

The exposure of white differentiated adipocytes from primary SVF to CL 316,243, a $\beta 3$ adrenergic agonist used as a positive control, results in an appropriate increase in *Ucp1* mRNA expression (for example, Figure 34A). This unique feature of the primary preadipocytes provides a model of white to beige adipocyte *trans*differentiation. Together, these data demonstrate that the differentiation of primary inguinal SVF cells from female mice provides a robust cell culture system, which is uniquely suited for studying the local effects of breast cancer and other components within the tumor microenvironment on white adipocyte browning.

We utilized this primary SVF *in vitro* cell culture system to explore whether the E0771 cell secretome or E0771 coculture with white adipocytes would induce WAT browning. To our surprise, *Ucp1* mRNA expression in white adipocytes exposed to

E0771 cancer conditioned medium or E0771 coculture was *decreased* relative to control white adipocytes (Figure 34 and Figure 36, respectively). Although not statistically significant, the trend of lower *Ucp1* expression in white adipocytes exposed to either the E0771 secretome or E0771 coculture was consistent with expression trends demonstrated in the immortalized preadipocyte cell culture system.

Ucp1 mRNA was used as the primary target in our studies, as UCP1 is the hallmark of thermogenic activity, and total *Ucp1* mRNA correlates well with total UCP1 protein in mouse models of cold-induced adipocyte browning [187]. Furthermore, western blotting of UCP1 in our experiments confirmed our *Ucp1* mRNA expression results on the protein level (Figure 35). This provided additional justification for the use of *Ucp1* mRNA expression. However, in the future, it would be helpful to gather additional data related to the *activity* of the increased UCP1 protein, for example, by measuring oxygen consumption rate and extracellular acidification rate using the Seahorse Analyzers (Agilent). This will require technical troubleshooting, as our initial attempts at using this technology were unsuccessful due to adipocyte detachment from cell culture plates even with the use of gelatin-coating.

While there were no changes in gene expression of the thermogenesis associated genes *Cidea* or *Ppargc1a*, white adipocytes cocultured with E0771 cells expressed significantly more *Tnfrsf9* (Figure 36B). White adipocytes exposed to conditioned medium had increased, although not statistically significant, *Tnfrsf9* (Figure 34B). *Tnfrsf9* has been found to be elevated in breast cancer xenografts [117], but within adipocytes, its expression changes often parallel *Ucp1* and *Ppargc1a* [188], which we did not observe in our results. TNFRSF9 (also known as CD137) promotes

the activation of a variety of immune cells [189], and can enhance natural killer cell-mediated destruction of breast cancer cells [190]. It is therefore possible that cross-talk between white adipocytes and the E0771 cell line induces an immune-eliciting/enhancing response by the adipocytes.

Lep and *Fasn* mRNA levels were significantly decreased in both treatment conditions compared to control differentiated white adipocytes, with no change in *Cfd* expression (Figure 34C and Figure 36C). In WAT, leptin expression is associated with lipid droplet size, so it was initially surprising that its expression is decreased in our experimental system with plentiful nutrients in the cell culture medium, but also seemed logical as this suggests lipolysis within the cells. Additionally, leptin is overexpressed in breast tumors [191] and contributes to tumorigenesis by playing a role in mesenchymal-epithelial transition and in regulating angiogenesis [192]. Additionally, leptin has been shown to induce Notch signaling leading to increased breast cancer aggressiveness [169]. Therefore, we speculate that the observed decrease in *Lep* expression by mature white adipocytes represents a protective response after exposure to E0771 conditioned medium or coculture. Multiple studies have shown that overexpression of fatty acid synthase, a key enzyme for *de novo* fatty acid synthesis, in breast cancer cells confers a more aggressive phenotype and allows for tumor growth and migration [193], so its decreased expression in adipocytes after treatment is consistent with a protective response. Additionally, transcription of *Fasn* is regulated, at least in part, by leptin [194], whose mRNA expression we show here as also being downregulated.

We also tested for genes associated with lipolysis, and found that white adipocytes exposed to E0771 cancer conditioned medium had no changes in

expression of *Lpl* (lipoprotein lipase) or *Mgll* (monoglyceride lipase), but expressed less *Lipe* (hormone sensitive lipase) compared to control white adipocytes (Figure 34D). However, expression of *Mgll* was significantly increased in white adipocytes cocultured with E0771 cells, while expression of *Lipe* and *Lpl* was decreased but not significantly (Figure 36D). Increased monoglyceride lipase, an enzyme involved in lipolysis to generate fatty acids, is implicated in progression of several cancer cell types, including aggressive breast cancer [195]. Our results suggest that E0771-adipocyte cross talk, not simply E0771 cell secretome components, may induce *Mgll* expression to provide substrates for E0771 cancer cell utilization. Further experiments are needed to target the cancer-adipocyte cross talk-related drivers of this process, and to determine if this process results in paracrine or endocrine signaling contributing globally to CAC.

In our last set of murine cell culture experiments in this chapter, we established primary SVF cell lines derived from BALB/c mice and used the 4T1 breast cancer cell line to test the reproducibility of our C57BL/6J-E0771 results in a different mouse strain. The 4T1 breast cancer cell line is an animal model for stage IV breast cancer, has a more metastatic phenotype than classic E0771 cells [196], and is commonly used for *in vitro* and *in vivo* BALB/c breast cancer studies [197] and allograft studies [198, 199].

4T1 conditioned medium exposure or coculture with BALB/c-derived mature adipocytes demonstrated very similar results to our E0771 experiments. *Ucp1* mRNA expression in white adipocytes exposed to 4T1 cancer conditioned medium or 4T1 coculture was *decreased* relative to control white adipocytes, although not statistically significant (Figure 37A and Figure 39A, respectively). Western blotting of UCP1 in these experiments confirmed, again, our *Ucp1* mRNA expression results on the protein

level (Figure 38). There were no statistically significant changes in thermogenic adipocyte-related gene expression, although there was a trend towards increased *Tnfrsf9*. As described above, it is still possible that an immune-eliciting/enhancing response by the adipocytes occurs in the presence of breast cancer. The only white adipocyte gene marker that was changed in these 4T1 experiments was a decrease in *Lep* expression after exposure to 4T1 cancer conditioned medium (Figure 37C). This is slightly different from the E0771 results, which demonstrated a decrease in *Lep* and *Fasn* in both treatment groups, but still suggests that an increase *Lep* expression by mature white adipocytes represents a protective response in the presence of breast cancer. No changes in any of the lipolysis-related genes occurs in the 4T1 experiments, which suggests that differences between the E0771 and 4T1 cancer cell lines and/or C57BL/6- and BALB/c-derived adipocytes may influence this aspect of adipocyte plasticity.

We then utilized human cell culture methods to test whether breast cancer directly induces WAT browning *in vitro*. First, we established the necessary cell culture conditions to induce white and beige differentiation from female human benign breast SVF cells, and found that human SVF cells need a low serum supplementation in order to maximize health and differentiation capacity (Figure 40). The medium supplementation has 2% FBS as opposed to 10% in the medium for murine cell culture, and includes additional supplementation of L-glutamine, ascorbic acid, recombinant human insulin, recombinant human FGF basic (fibroblast growth factor basic, also known as FGF2), and hydrocortisone hemisuccinate. Many cells are cultured in low or serum-free medium either at baseline or for particular experimental designs [200], and

decreased serum here may be helpful here by increasing sensitivity of differentiating cells to induction and/or maintenance medium. While L-glutamine and ascorbic acid are both included in the DMEM medium used in our lab for most cell culture, they are supplemented at much higher concentrations here, with approximately 3 times more L-glutamine and 8 times more ascorbic acid. Ascorbic acid is a water soluble molecule, and additional amounts may be helpful as it acts as an antioxidant, electron donor for several enzymes, and helps to protect against peroxidation of unsaturated fatty acids [201]. Insulin is included in all induction and maintenance mediums, but is not in pre-differentiation medium for mice; we suspect human SVF may require the additional signaling that insulin provides to stimulate healthy cell proliferation before differentiation. Hydrocortisone hemisuccinate is a water-soluble immunosuppressant, and we suspect that it aids in human cell culture by dampening any autocrine or paracrine signaling that may strain the cell before or during differentiation. Lastly, FGF2 activates tyrosine kinase activity in vivo [202], and in murine adipogenesis studies using 3T3-L1 cells, the nonreceptor tyrosine kinase c-Abl (Abelson murine leukemia viral oncogene) is critical for initiation of differentiation [203]; we therefore expect that the addition of FGF2 aids in priming our SVF for differentiation and in promoting differentiation once it begins. Once optimized, our conditions yielded differentiation to mature white and beige adipocytes with appropriate differences in lipid accumulation and *UCP1* expression (Figure 41). We then used this model to test the effects of two human breast cancer cell lines with different receptor expression and aggressiveness on white adipocyte plasticity: MCF-7 (estrogen receptor positive) and MDA-MB-231 (triple negative, more aggressive phenotype) [196].

Using SVF derived from benign white breast adipose tissue from a 27 year old woman with a history of diabetes mellitus, we show, similar to that in mice (Figure 28), that most significant changes occur when differentiating cells are exposed to cancer conditioned medium during periods of maintenance or throughout induction and maintenance phases (Figure 42). *UCP1* mRNA was significantly *increased* in maturing adipocytes exposed to conditioned medium from MDA-MB-231 cells. In these adipocytes treated with MDA-MB-231 conditioned medium, *SLC27A1* was significantly increased, while *PPARG* and *PPARGC1A* were significantly decreased. While not significant, maturing adipocytes exposed to MDA-MB-231 conditioned medium expressed about 4-fold less *EAR2*, and 2-fold less *OPLAH* and *PDK4* (Figure 42). Interestingly, these changes were not observed in maturing adipocytes exposed to conditioned medium from MCF-7 cells; instead, these adipocytes expressed similar or less mRNA (though not significant) for all of these genes compared to controls.

SLC27A1 encodes SLC27A1, more commonly known as fatty acid transport protein 1 (FATP1), helps to control fatty acid uptake into cells [204]. Its expression has been shown to be inversely related to BMI and adipocyte size, and increases as human subjects lose weight [205]. Although its closely related family member SLC27A4 (FATP4) has just recently been shown to be increased in human breast cancer tissues and increases free fatty acid uptake in the MDA-MB-231 cell line, in the same study, higher SLC27A1 expression was correlated with increased survival [206]. Therefore, its increased expression by mature adipocytes that were exposed to MDA-MB-231 cells likely manifests as a protective response by the adipocytes, perhaps to change substrate utilization to the demise of the tumor cells.

PPARG in mice has been shown to be necessary for brown adipose tissue function and intact beta-3 adrenergic signaling [207], as it is a transcriptional regulator on the UCP1 promoter region. In breast cancer cells, PPARG is overexpressed and can induce cell differentiation, although it is contested whether this exerts a positive or negative effect on cancer progression [208]. A 2005 study found a correlation between PPARG expression and estrogen receptor beta expression and that it is a favorable prognostic factor for disease free survival [209]. Our results demonstrate significantly decreased *PPARG* expression in the adipocytes treated with MDA-MB-231, while we observed a mild decrease – though not significant – in expression after treatment with MCF-7 conditioned medium. Since the MCF-7 cell line is estrogen receptor positive, and MDA-MB-231 is not, this could perhaps explain the difference in the degree of decreased *PPARG* expression. Breast cancer secreted microRNAs, such as miR-155, downregulate PPARG expression and lead to metabolism reprogramming in mature adipocytes contributing to cancer associated cachexia [210]. While we did not test for the presence of specific factors such as miR-155, it is possible that they are differentially expressed in different breast cancer cell lines and may contribute to the observed decrease in *PPARG* expression.

Although *UCP1* was significantly increased in the MDA-MB-231 conditioned medium treatment group, *PPARGC1A* was significantly decreased. This was initially surprising, as PPARGC1A substantially increases PPARG activity on the UCP1 promoter and is known to be involved in WAT browning in non-cancer-related studies [211, 212]; however, it is not essential. The decreased *PPARGC1A* may therefore be a response to the increased *UCP1* expression, providing negative feedback to quench the

maladaptive increase in *UCP1*. Other genes that are associated with classical brown adipose tissue such as *OPLAH* [117] and WAT browning such as *PDK4* and *EAR2* [213, 214] were decreased, though not significantly, after exposure to MDA-MB-231 cells during differentiation. This further suggests that although *UCP1* is increased in these cells, other cell signaling processes are acting to downregulate that effect to return to 'homeostasis.'

When comparing lipid droplet sizes in these adipocytes, our results demonstrate that cancer conditioned medium from either human breast cancer cell line results in significantly decreased lipid droplet size in mature adipocytes, regardless of when the exposure occurred during differentiation. Interestingly, when exposure occurred during maintenance or during induction *and* maintenance, cells exposed to cancer conditioned medium from MDA-MB-231 cells had a more significant decrease in lipid droplet size than the MCF-7 conditioned medium-treated group. These results further support our mRNA expression data, which suggest a differential response in adipocyte differentiation to MDA-MB-231 versus MCF-7 secretomes. This further supports the mRNA expression data for genes such as *SLC27A1*, which was significantly increased and is known to increase with decreasing adipocyte size [205]. While we had different thermogenic adipose tissue-related gene expression changes, the decreased lipid droplet sizes in both exposure groups correlate with the 'cancer associated adipocyte' phenotype that has been recently described in breast cancer [116]. An increased amount of lipid droplets has been shown to be associated with increased aggressiveness of cancer using MCF-7 and MDA-MB-231 cells [215], but it is unclear whether the lipid accumulation is a result of cancer-specific process or of local WAT

changes and process. It would be interesting to test for genes related to lipid metabolism and lipolysis to better understand the functional meaning of the changes that we and others have noted in both adipocyte and breast cancer cells. Additionally, cancer-associated adipose tissue has been shown to produce a protein secretome with inflammatory characteristics [216], which we did not explore in our studies. It would be interesting to test for these changes as well to better understand the complicated role of inflammation and how it relates to breast cancer tumorigenesis and adipocyte-specific changes in breast cancer.

Lastly, we tested the effects of these two human breast cancer cell lines' conditioned mediums on mature white adipocytes in an SVF cell line derived from a different human patient (this time, a 51 year old woman with no history of diabetes mellitus) during differentiation and after maturation for 24 hours. Interestingly, our results suggest that 24 hours of MCF-7 conditioned medium exposure is able to increase *UCP1* mRNA expression by about 4-fold, while MDA-MB-231 conditioned medium *decreased* *UCP1* mRNA expression by more than 8-fold (Figure 44C). When replicating the conditions using the first SVF cell line, exposure of this different SVF cell line to conditioned medium during induction and maintenance phases of differentiation resulted in significant *decreases* in *UCP1* mRNA expression compared to control (Figure 44B). These results are in contrast to the results using the first SVF cell line, which suggested that MDA-MB-231 conditioned medium caused an *increase* in *UCP1* expression (Figure 42).

These conflicting results make it challenging to establish conclusive conclusions regarding the direct effects of MCF-7 and MDA-MB-231 secreted factors on white

adipocyte plasticity. It is entirely possible that the difference between *UCP1* expression in the first SVF cell line (Figure 42) and the second (Figure 44) are due to differences in the women in which these cells were isolated from. It is well established that phenotypic properties of tissue donors are at least partially retained when their cells are cultured *in vitro* [217]. Therefore, the age difference (27 versus 51 years of age, respectively), difference in diabetes status (positive versus negative, respectively), or difference in other factors unknown to us such as body weight, between the two women whose normal breast WAT was used for SVF isolation may be responsible for the differences we have observed in our experiments. This suggests that individual characteristics may render their differentiating and mature white adipocytes more or less resilient to cancer-mediated adipocyte changes. Additional experiments using a larger sample of human-derived SVF cells lines would be needed for further exploration.

Furthermore, there is clearly a differential response within an individual SVF cell strain to different breast cancer cell lines. There are a host of differences including and beyond those in the estrogen, progesterone, and HER2 receptor statuses between the MCF-7 and MDA-MB-231 cell lines. Differences in angiogenic factors in the secretomes of MCF-7 and MDA-MB-231 cells were found in a 2016 xenograft study [218]. Estrogen increases oxidative stress in ER-positive cell lines such as MCF-7 cells to a significantly greater extent than ER-negative lines such as MDA-MB-231 [219], and increased oxidative stress and inflammation may be involved in adipocyte changes in breast cancer. For example, insulin-like growth factor I receptor is increased in estrogen-receptor-positive breast cancers such as MCF-7, resulting in PI-3K/Akt pathway activation that is not found in non-estrogen-receptor-positive breast cancers such as

MDA-MB-231 [220]. PPAR α activation is higher in MDA-MB-231 cells than in MCF-7 cells with implications related to increased proliferation and tumorigenesis [221]. Other cell lines, including these, with different characteristics should be used to provide additional understanding and mechanisms for these differences.

In both these murine and human *in vitro* experiments, we must consider additional limitations to the experimental design and the impact of these on our results. The *in vivo* environment contains additional cell types not included in these more simplistic *in vitro* models. Additionally, the two-dimensional nature of our cell culture models is inherently different than the three-dimensional *in vivo* environment, which limits our ability to fully replicate the interactions and processes occurring *in vivo*. For example, our *in vitro* model does not include hypoxic conditions that are known to be present in tumors *in vivo* [134], angiogenesis which can regulate thermogenesis *in vivo* [222], nor elevated glycolysis which has been shown to occur in cancer and contribute to cancer-associated cachexia [223, 224]. Future experiments should consider three-dimensional cell culture systems (reviewed by [225]) with multiple cell types to allow for more robust conclusions.

4.4 Conclusion

Together, our results show that cancer conditioned medium and coculture modify the overall adipocyte gene expression pattern, but do not alter thermogenic gene expression in murine *in vitro* studies. This supports prior literature regarding non-thermogenic changes in cancer, and challenges those related to cancer-induced WAT browning; however, since the *in vivo* environment is multifaceted, the complexities of

the cells and signaling within the tumor microenvironment should be explored further before completely ruling out WAT browning effects from the E0771 or 4T1 breast cancer cell lines. In our human *in vitro* studies, our results using MCF-7 and MDA-MB-231 breast cancer cell lines are more ambiguous, highlighting the importance of individual variability and susceptibility to breast cancer-mediated changes. Our results, especially in our murine experiments, suggest that the local WAT browning observed in the previous chapter are not mediated by factors directly secreted by, or released as a result of cross talk with, the E0771, 4T1, or LLC cancer cell lines. This suggests that local WAT browning is the result of interactions with other cells and factors within the tumor microenvironment. We explore this in the subsequent chapter.

Chapter 5: The Role of Tumor Microenvironment in Cancer-Mediated White Adipose Tissue Browning and Plasticity

5.1 Rationale

In the previous chapters, we demonstrate that WAT browning occurs locally *in vivo* (Chapter 3), but that cancer conditioned medium and coculture do not alter thermogenic gene expression in murine *in vitro* studies (although they do modify the overall adipocyte gene expression pattern, Chapter 4). These results suggest that the tumor microenvironment is involved in this process, and we ask here whether several components – interleukin 6, tumor lysate, and a host of immune cell populations – are involved in WAT browning and adipocyte plasticity.

5.2 Results

5.2.1 IL6 is minimally secreted by selected murine cancer cell lines, and exposure of differentiated white adipocytes to exogenous IL6 does not increase Ucp1 gene expression

Considering that tumor-derived IL6 has been implicated in other models of cancer-related WAT browning, we asked whether our ER+ murine breast cancer E0771 cell line secreted IL6 into the conditioned medium. Our results demonstrate that the E0771 cells produce IL6, but at much lower levels than peritoneal cavity inflammatory cells as measured by intracellular flow cytometry (Figure 45A,B) and qPCR (Figure 45C). We also demonstrate similar expression patterns in other cancer cell lines: 4T1 breast cancer cells and LLC cells, with LLC cells expressing significantly less *Il6* mRNA than stimulated murine peritoneal cavity cells (Figure 46).

As IL6 may come from a variety of cell types within the tumor microenvironment *in vivo*, we next sought to determine whether our mature adipocytes *in vitro* have the necessary receptor machinery – interleukin 6 signal transducer (IL6ST, also referred to as glycoprotein 130, or GP130) and membrane-bound interleukin 6 receptor alpha (IL6RA) – for IL6 signaling to occur. Flow cytometry demonstrates that differentiated white and beige adipocytes express abundant IL6ST (aka GP130, or CD130), as evidenced by increased staining relative to isotype control (Figure 47A). Gene expression analysis for *Il6st* supports the flow cytometry data: white and beige adipocytes express at least the same amount of *Il6st* (encodes GP130) mRNA compared to stimulated peritoneal cavity cells (Figure 47B). Interestingly, white and beige adipocytes do *not* express IL6RA (CD126) on their cell membranes, evidenced by less flow cytometry staining than isotype control (Figure 47C). *Il6ra* mRNA expression is also decreased in white and beige adipocytes relative to stimulated peritoneal cavity cells (Figure 47D).

With the above information, we investigated if exogenous IL6 exposure, with or without supplementation with soluble IL6RA, would induce gene expression changes indicative of WAT browning *in vitro*. Increased *Socs3* (suppressor of cytokine signaling 3) gene expression (Figure 48A) and increased phosphorylated STAT3 protein at the tyrosine 705 residue (Figure 48B) following 30 minutes of exposure to IL6 and IL6+IL6RA demonstrate the bioactivity of IL6 and IL6RA in our experimental model. To our surprise, exposure of white adipocytes to IL6 or IL6+IL6RA for three days did not increase *Ucp1* mRNA expression but instead resulted in a downward trend in expression (Figure 49A). A similar trend in *Cidea* expression was observed (Figure

49B). The IL6+IL6RA treated group expressed significantly more *Tnfrsf9* mRNA compared to control white adipocytes but had no changes in *Ppargc1a* expression (Figure 49B). *Lep* mRNA expression was significantly decreased with IL6+IL6RA treatment, but no changes were observed for *Cfd* or *Fasn* (Figure 49C). No change in any of the measured lipolysis markers was observed in the white adipocytes treated with IL6+IL6RA (Figure 49D).

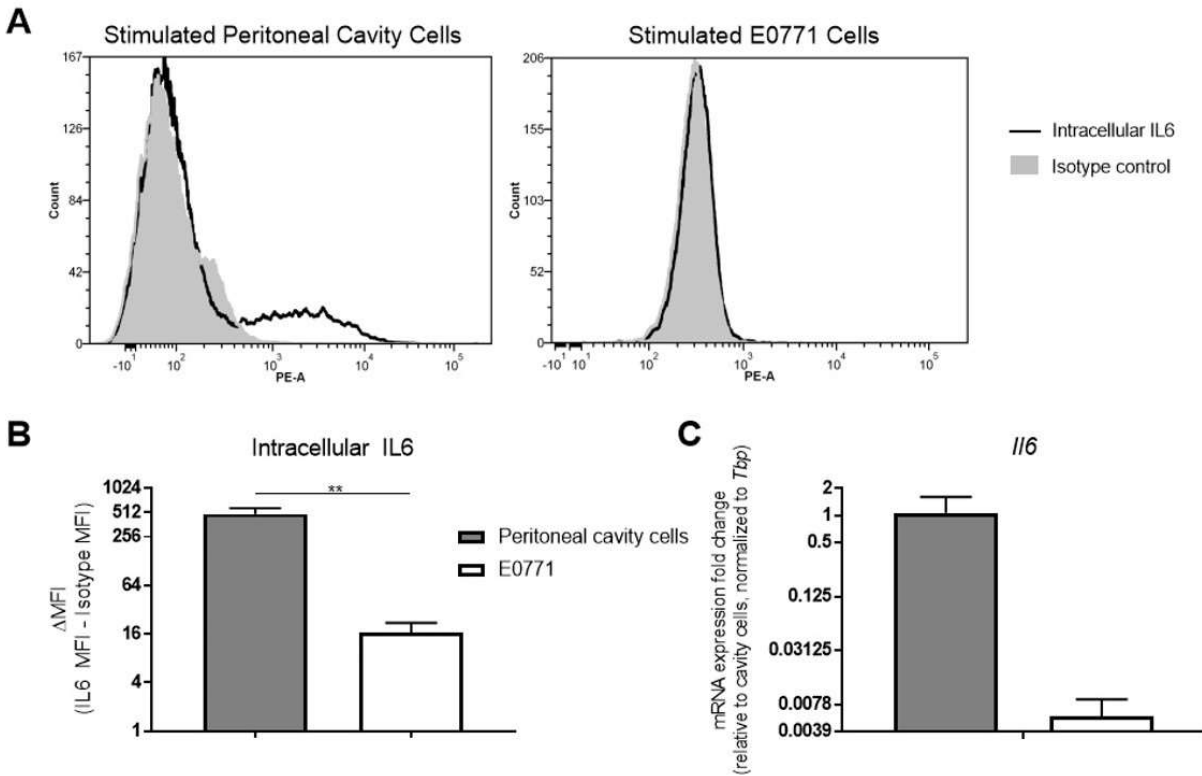


Figure 45. E0771 cells do not produce excessive IL6

Peritoneal cavity cells and E0771 cells were stimulated to induce endogenous IL6 production as described in methods. (A) Intracellular flow cytometry curves showing staining data for IL6 stain and isotype control. (B) Quantification of intracellular IL6 from flow cytometry MFIs. (C) Quantitative PCR for *Il6* mRNA expression. Please note y-axis scale in (B) and (C) is log2. $n = 2$ for peritoneal cavity cells; $n = 3$ for E0771 cells. Statistical tests: (B) Unpaired t-test, (C) Mann-Whitney test. MFI = Mean Fluorescent Intensity.

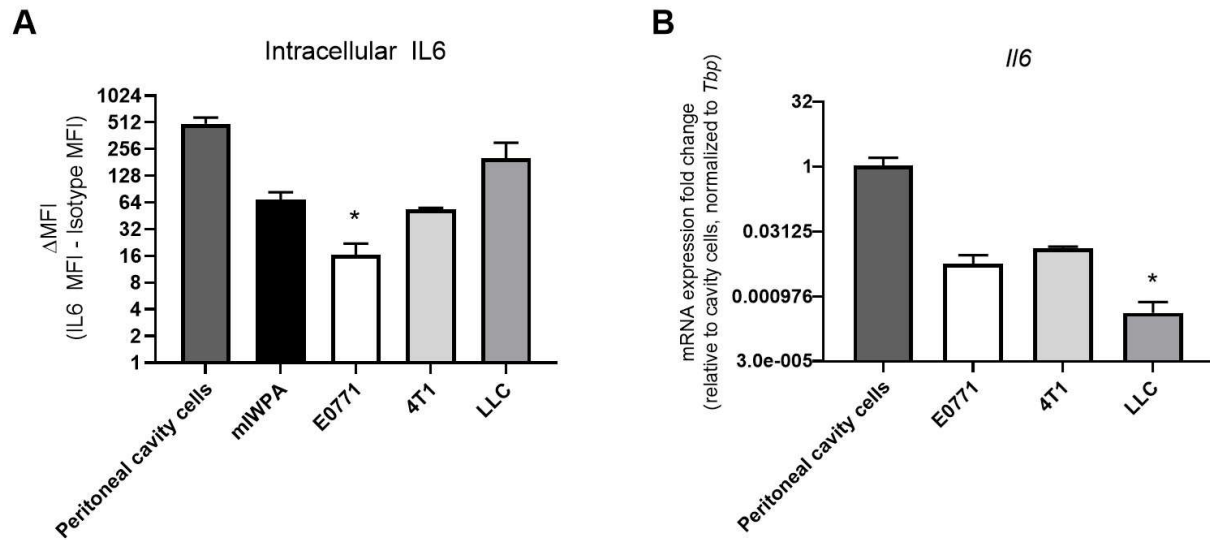


Figure 46. E0771, 4T1, and LLC cells do not produce excessive IL6

Peritoneal cavity cells, immortalized SVF preadipocytes ('mWPA' in image), and different cancer cells (E0771, 4T1, and LLC) were stimulated to induce endogenous IL6 production as described in methods. (A) Quantification of intracellular IL6 from flow cytometry MFIs. (B) Quantitative PCR for IL6 mRNA expression. Please note y-axis scale in (B) is log2. n = 2 for peritoneal cavity cells; n = 3 for all other cells. Statistical tests: Kruskal-Wallis (nonparametric ANOVA) test with Dunn's test for multiple comparisons. MFI = Mean Fluorescent Intensity.

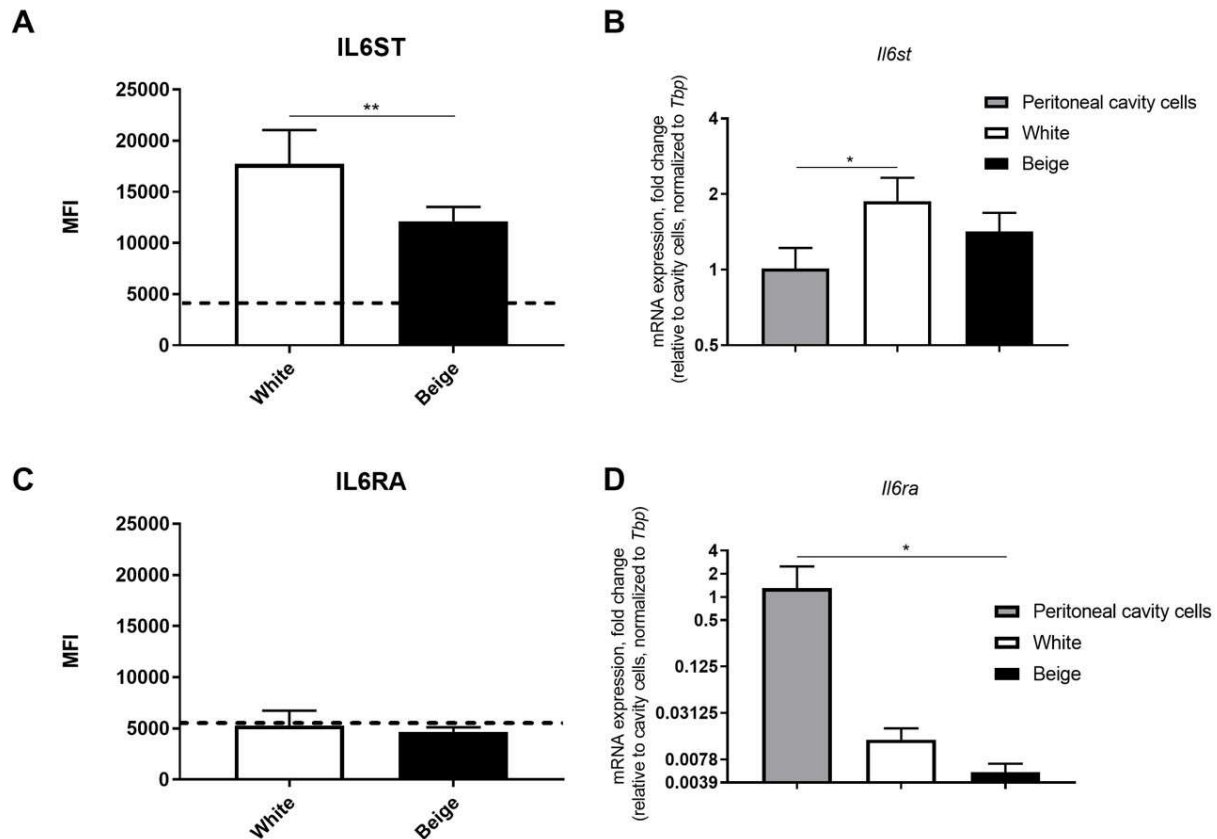


Figure 47. Differentiated white and beige adipocytes express abundant IL6ST, but not IL6RA

Flow cytometry for (A) cell-surface IL6ST (GP130, CD130) or (C) IL6RA (CD126) in white and beige adipocytes. Dashed line represents average MFI staining for each respective isotype control. Quantitative PCR for (B) *Il6st* (Gp130) or (D) *Il6ra* (Cd126) for peritoneal cavity cells and white and beige adipocytes. $n = 2$ for peritoneal cavity cells; $n = 5$ SVF cell lines for white and beige adipocytes. Please note y-axis scale in (B) and (D) is log2. Statistical tests: (A) and (C) Mann-Whitney test, (B) and (D) Kruskal-Wallis and Dunn's multiple comparisons tests. MFI = Mean Fluorescent Intensity.

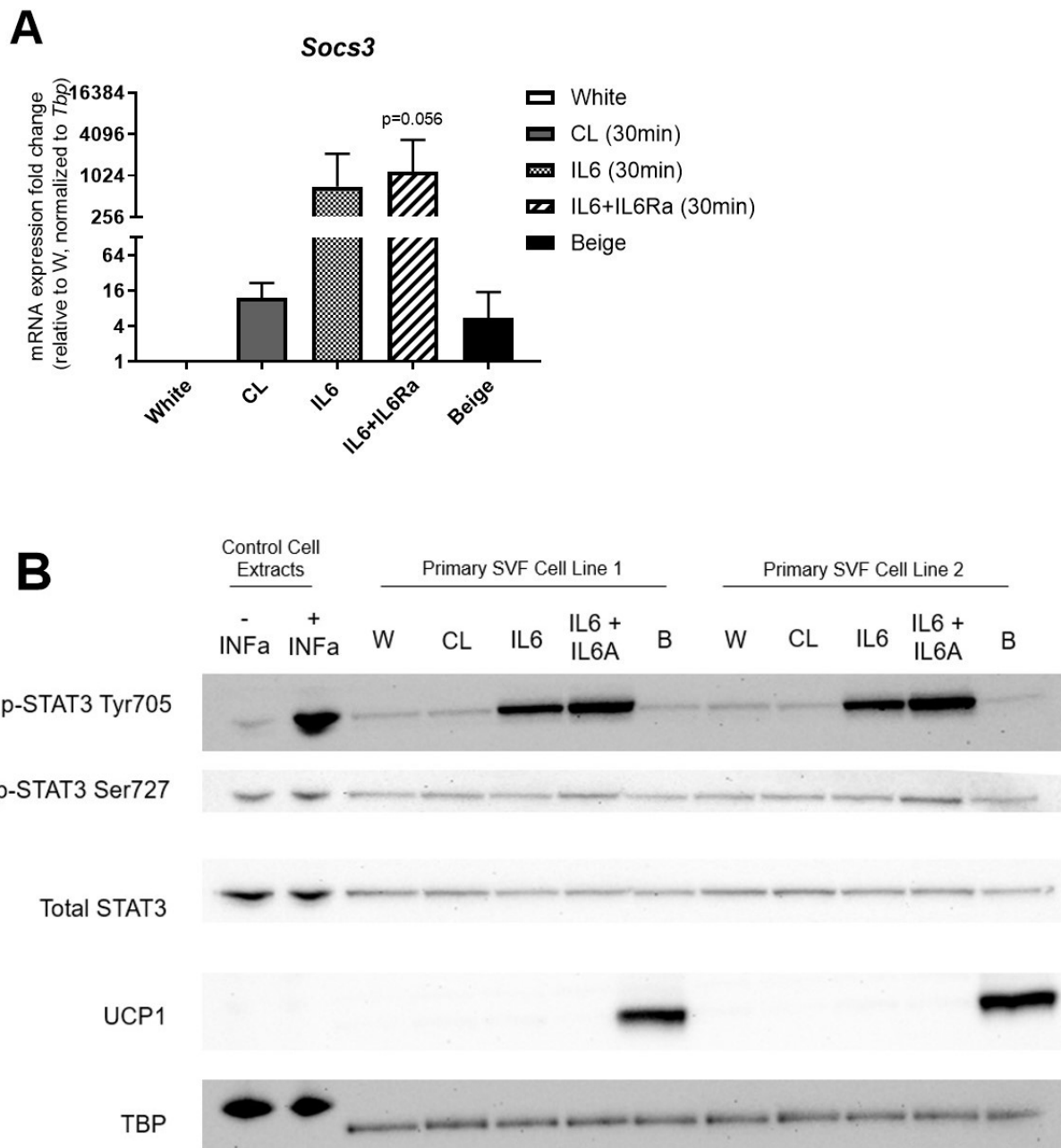


Figure 48. Both IL6 and IL6+IL6RA treatment induces STAT3 signaling in mature white adipocytes, with IL6+IL6RA treatment inducing most robust signaling.

Differentiated white adipocytes were treated for 30 minutes with CL316,243 (noted as 'CL' in images), IL6 40 ng/mL + IL6RA 200 ng/mL (noted 'IL6+IL6RA' in images), and controls as described in methods. (A) Quantitative PCR for Socs3. Please note y-axis scale is log2. n = 3 primary SVF cell lines. Error bars = standard deviation. Statistical tests: Friedman and Dunn's multiple comparisons tests. (B) Western blot of phosphorylated STAT3 ('p-STAT3' with different residues indicated in image), total STAT3, UCP1, and TBP protein.

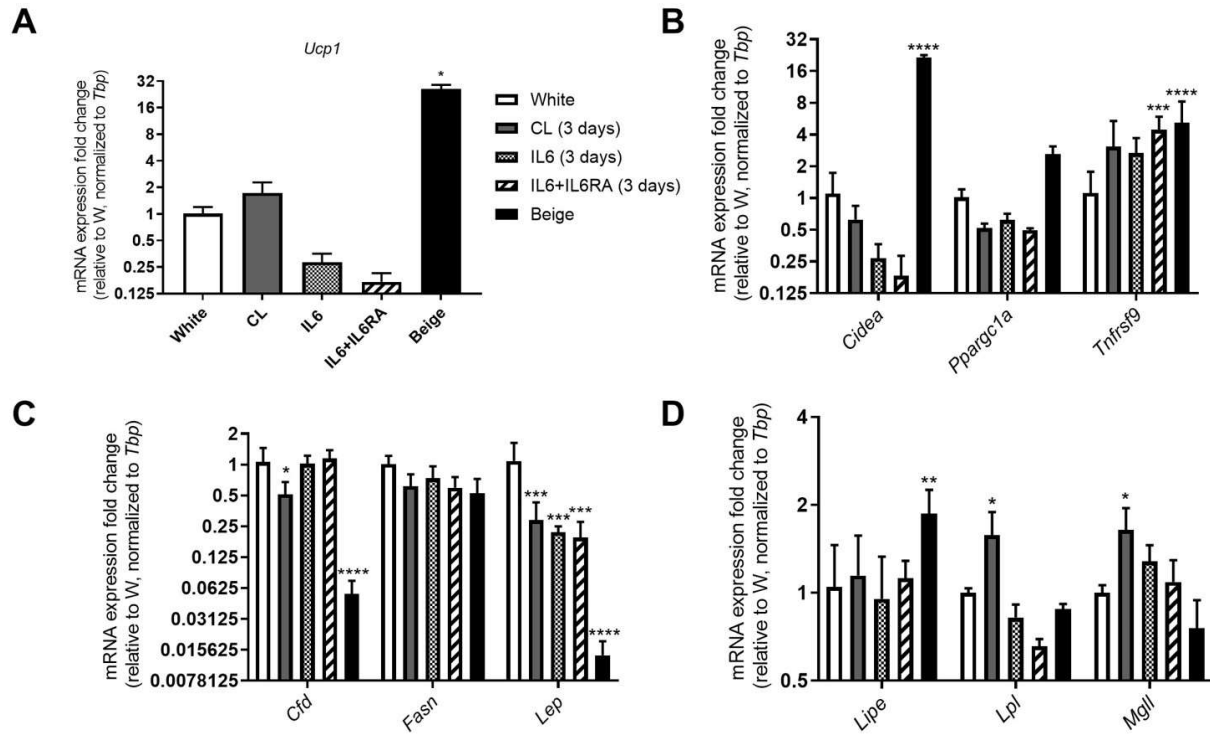


Figure 49. Neither exogenous IL6 or IL6+IL6RA administration causes WAT browning in vitro, but alters white adipocyte mRNA expression

Differentiated white adipocytes were treated for 3 days with CL316,243 (noted as 'CL' in images), IL6 40 ng/mL (noted as 'IL6' in images), IL6 40 ng/mL + IL6RA 200 ng/mL (noted 'IL6+IL6RA' in images), and controls as described in methods. Quantitative PCR for (A) *Ucp1*, (B) markers of beige adipocytes, (C) markers of white adipocytes, (D) markers of lipolysis. Please note y-axis scale is log2. n = 3 primary SVF cell lines. Error bars = standard deviation. Statistical tests: (A) Kruskal-Wallis and Dunn's multiple comparisons tests, (B), (C), and (D) Two-way ANOVA and Dunnett's multiple comparisons tests.

5.2.2 Preliminary data: potential role of tumor lysate and/or tumor-isolated CD11b+ cells on WAT browning

After demonstrating that IL6 alters some adipocyte-related gene expression, but does not directly mediate WAT browning, we asked if the immune system component of the tumor microenvironment is involved in these processes. Using our primary inguinal SVF adipogenesis cell culture system, we exposed mature white adipocytes from two different SVF cell strains to a variety of conditions (refer to methods section 2.10) including tumor lysate and CD11b+ cells from several allograft and spontaneous murine breast tumors for 3 days. Mature beige adipocytes express appropriately more *Ucp1* than white adipocytes (Figure 50A, C); however, results exposure to experimental conditions are quite variable: some of the tumor lysate conditions altered *Ucp1* mRNA expression in white adipocytes positively or negatively, and to varying degrees, depending on the SVF strain (Figure 50B, D).

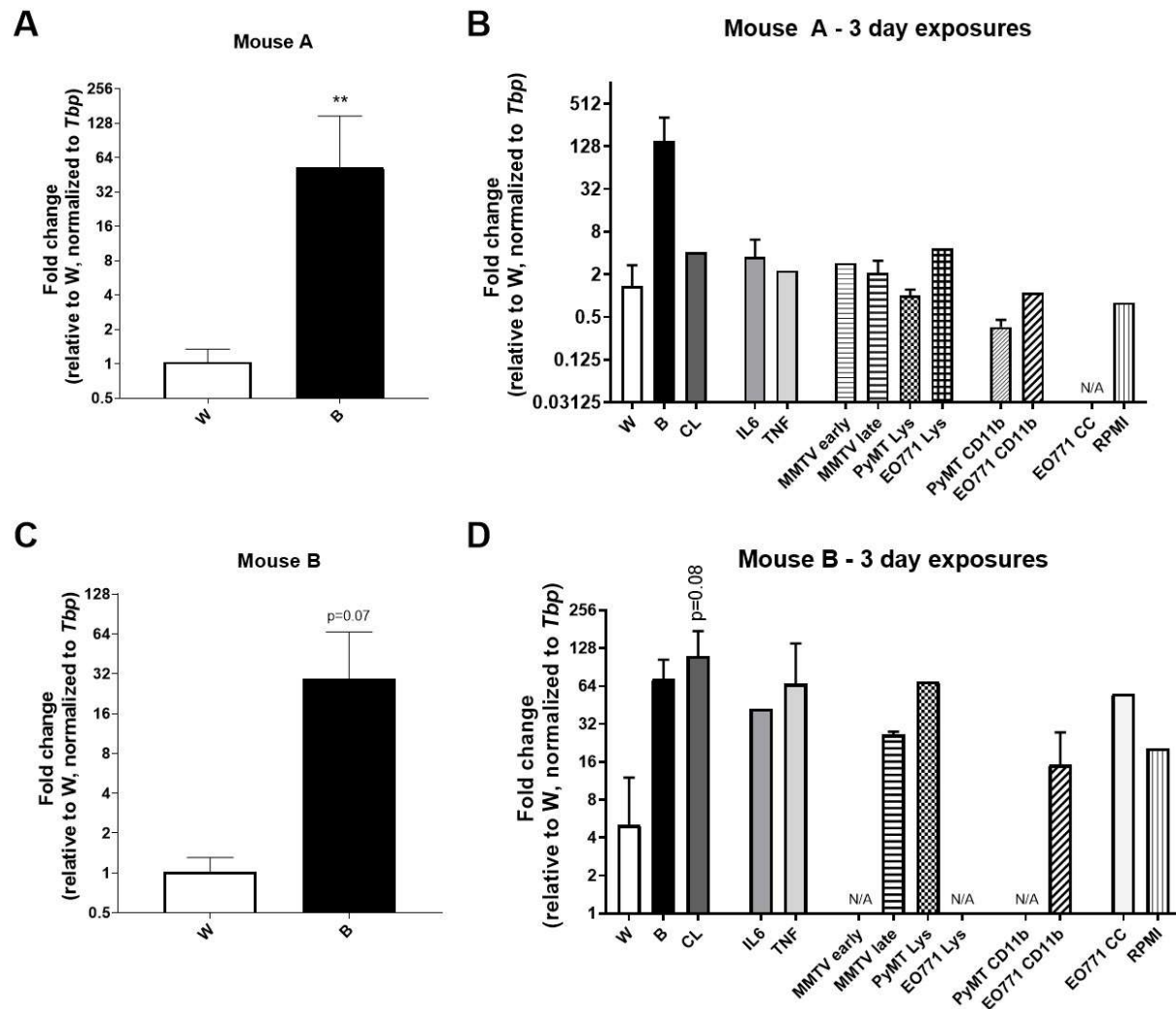


Figure 50. Tumor lysate and tumor-derived CD11b+ cells alter *Ucp1* expression in white adipocytes in vitro

Quantitative PCR for *Ucp1* mRNA expression. (A) and (C) Primary SVF were differentiated to mature white and beige adipocytes. (B) and (D) Mature white adipocytes were exposed to CL 316,243 ('CL' in image), IL6, TNF, and various tumor lysates ('MMTV early,' 'MMTV late,' 'PyMT Lys,' and 'EO771 Lys' in image) and CD11b cells ('PyMT CD11b' and 'EO771 CD11b' in image) for 3 days as described in methods. Please note y-axis scales are log2. (A) and (C) n=6 wells; (B) and (D) n = 2-3 wells per group. Statistical tests: (A) and (C) Mann-Whitney test; (B) and (D) Kruskal-Wallis (nonparametric ANOVA) test with Dunn's test for multiple comparisons.

5.2.3 Preliminary data: immune cell populations shift in adipose tissue in mice with breast cancer

We next wanted to explore which immune cell populations within the breast tumor microenvironment may be most important. After injecting wild-type and *Rag1* deficient (*Rag1*^{-/-}) BALB/c mice with 4T1 breast cancer cells (refer to methods section 2.11), we found that there were no significant changes in body weight in the mice between inoculation with cancer cells or saline control and euthanization (Figure 51A), nor were there any significant changes in rectal temperature at time of euthanization (Figure 51B). We harvested inguinal WAT (where the tumor cells were injected) from these mice to look at differences in gene expression of several key adipocyte markers between naïve mice and mice with tumor.

Compared to wild-type naïve mice, inguinal WAT from wild-type mice with 4T1 tumors expressed increased, though not significantly, *Ucp1*, *Cidea*, *Prdm16*, and *Ppargc1a* mRNA (Figure 51C, gray bars). In the *Rag1*-deficient mouse strain, compared to the *Rag1*^{-/-} naïve mice, inguinal WAT from *Rag1*^{-/-} mice with 4T1 tumors did not have changes in *Ucp1* expression, but had increased *Cidea* and *Ptk2b* expression and decreased *Ppargc1a* expression – although again, none of these changes were statistically significant (Figure 51C, black bars).

We analyzed adipose tissue (inguinal WAT, axillary WAT, gonadal WAT, and interscapular BAT) and the tumors themselves via flow cytometry to quantify immune cell populations. There were no trends or changes in percentage of CD45⁺ cells between naïve or mice with tumor in either mouse strain (Figure 52A). Neutrophils comprised a larger percentage of all live cells in all adipose tissue pads in both mouse

strains with tumor (Figure 52B), and there was a less impressive increase in dendritic cell population in the right inguinal, axillary, and gonadal fat pads in mice with tumor (Figure 52C). Conversely, in most adipose tissue pads, T cell and B cell populations were larger in naïve (non tumor-burdened) mice (Figure 52D, E). There was a trend toward an increased pan-macrophage population in many of the WAT pads of mice with tumor, especially in the right inguinal WAT where 4T1 cells were injected (Figure 53A). Looking at the subpopulations of macrophages, there appears to be an increase in major histocompatibility complex class II (MHCII)/lymphocyte antigen 6 complex (Ly6C)⁻ and MHCII⁻/Ly6C⁺ macrophages, a decrease in MHCII⁺/Ly6C⁻ macrophages, and no clear change in MHCII⁺/Ly6C⁺ macrophages (Figure 53B-E).

When we analyzed the percentages of immune cell population of all living *and* CD45⁺ cells, the trends just described above are even more apparent: mice with 4T1 cancer cells, regardless of wild-type or *Rag1*^{-/-} strain, have a larger percentage of neutrophils and dendritic cells (Figure 54A-B), a smaller percentage of T cells and B cells (Figure 54C-D), and similar or increased percentage of pan-macrophages (Figure 54E) in all or many adipose tissue pads. The increase in pan-macrophages is, again, most striking in the right inguinal WAT (Figure 54E, Figure 55A show same information). Of the live, CD45⁺, pan-macrophage cell population, we continue to see an increase in MHCII⁻/Ly6C⁻ and MHCII⁻/Ly6C⁺ macrophages, a decrease in MHCII⁺/Ly6C⁻ macrophages, and no clear change in MHCII⁺/Ly6C⁺ macrophages in both wild-type and *Rag1*^{-/-} mice with 4T1 breast cancer cells (Figure 55B-E).

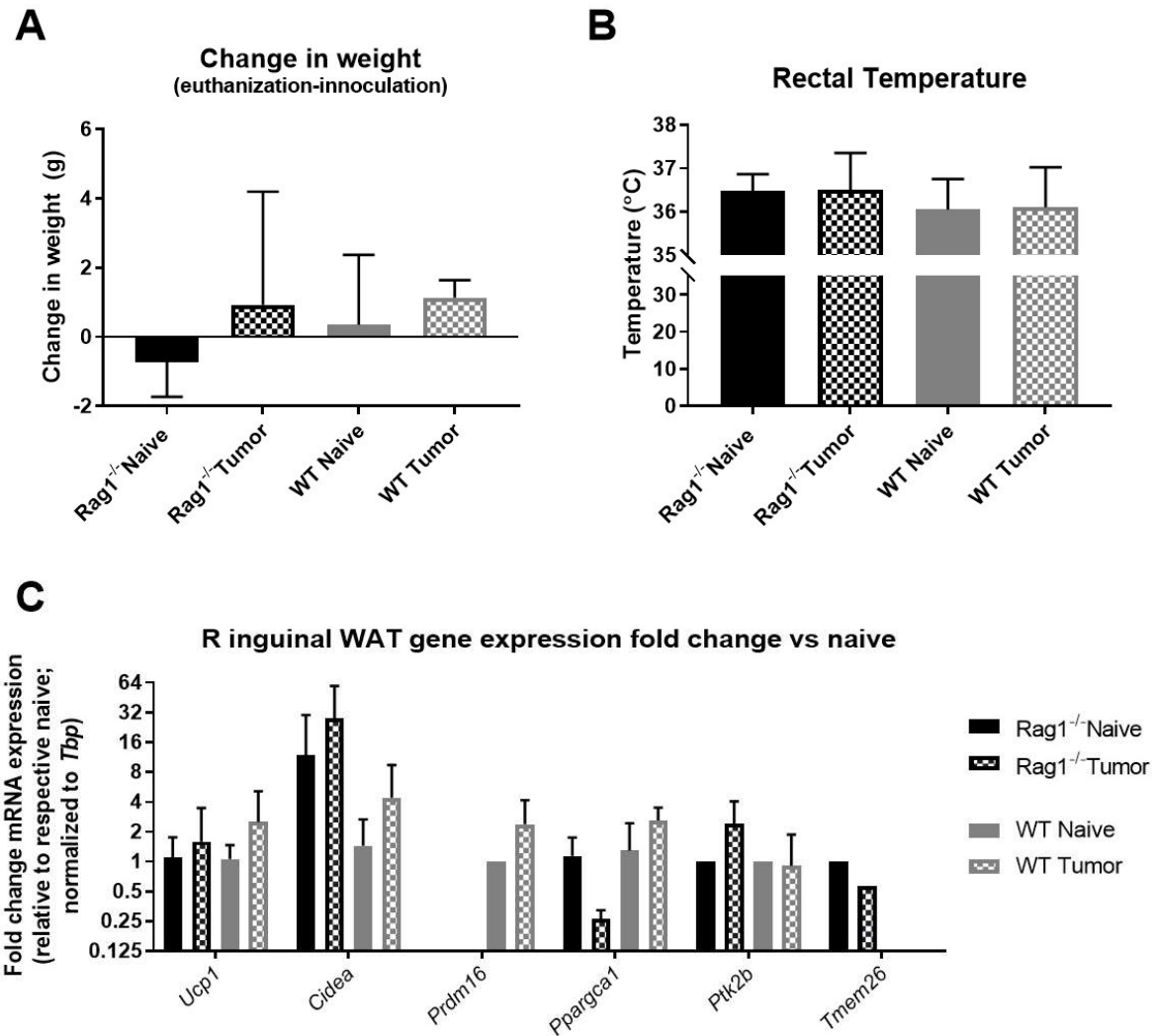


Figure 51. Weight, temperature, and *Ucp1* mRNA expression data from 4T1 allograft studies in wild-type and *Rag1*^{-/-} mice

Wild-type ('WT' in image) and *Rag1*^{-/-} mice of both male and female sex were inoculated with 4T1 cells or injected with control PBS and euthanized after tumor growth as described in methods. (A) Change in weight between inoculation and euthanization, (B) rectal temperature of mice at time of euthanization, (C) Quantitative PCR for several genes in right inguinal WAT (where injections occurred). For (C), fold changes were calculated with respect to each genotype's naïve expression levels, and please note y-axis scale is log2. For all groups, n=2-5. Statistical tests: (A) and (B) Kruskal-Wallis (nonparametric ANOVA) test with Dunn's test for multiple comparisons; (C) two-way ANOVA with Tukey's multiple comparisons test

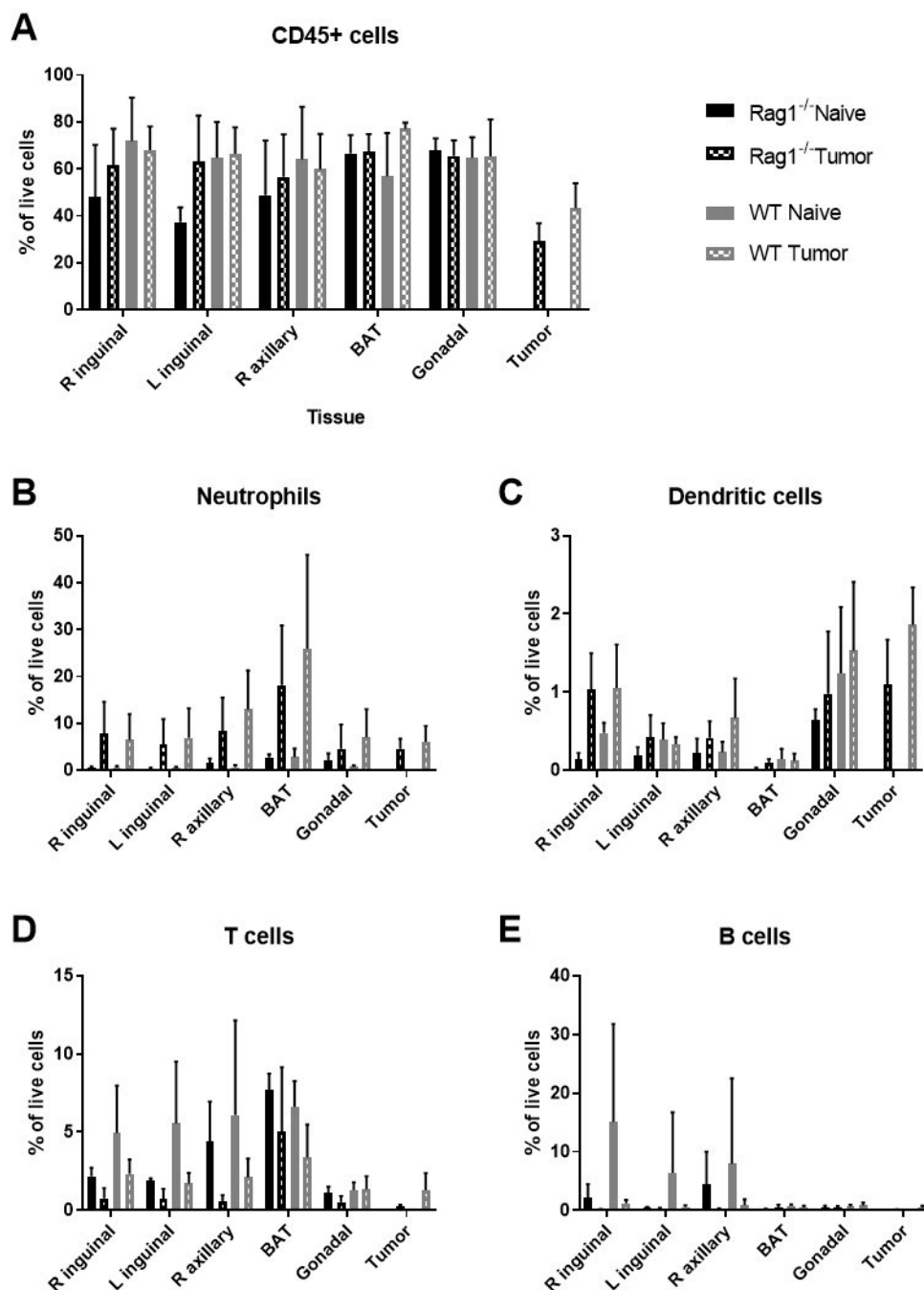


Figure 52. Various immune cell population percentages of live cells from 4T1 allograft studies in wild-type and *Rag1*^{-/-} mice

Wild-type ('WT' in image) and *Rag1*^{-/-} mice of both male and female sex were inoculated with 4T1 cells or injected with control PBS and euthanized after tumor growth as described in methods. Flow cytometry results for (A) CD45+ cells, (B) Neutrophils, (C) Dendritic cells, (D) T cells, (E) B cells. For all groups, n=4-5 mice.

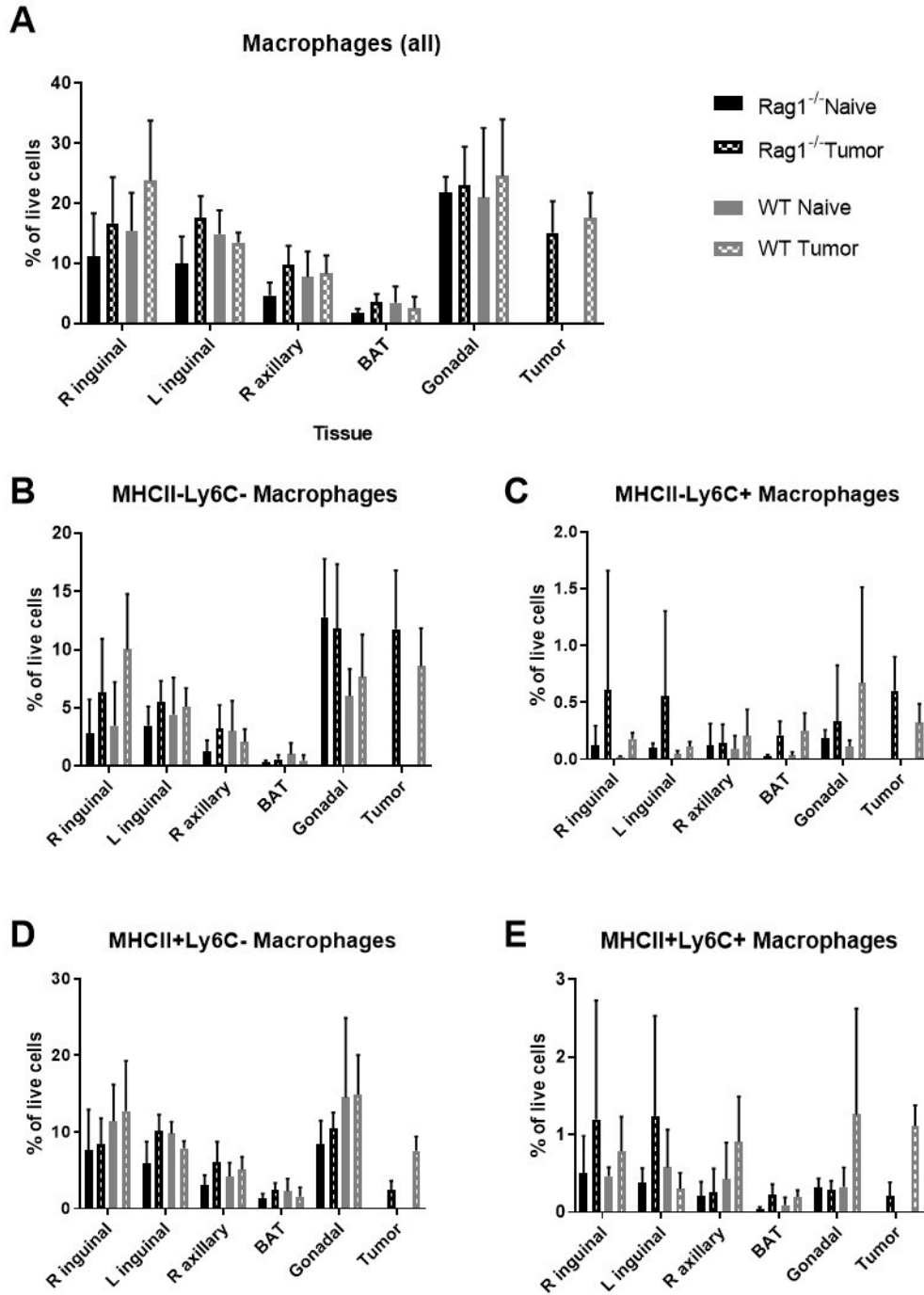


Figure 53. Macrophage population percentages of live cells from 4T1 allograft studies in wild-type and Rag1^{-/-} mice

Wild-type ('WT' in image) and Rag1^{-/-} mice of both male and female sex were inoculated with 4T1 cells or injected with control PBS and euthanized after tumor growth as described in methods. Flow cytometry results for (A) Pan-macrophages, (B) MHCII⁻/Ly6C⁻, (C) MHCII⁻/Ly6C⁺, (D) MHCII⁺/Ly6C⁻, (E) MHCII⁺/Ly6C⁺. For all groups, n=4-5 mice.

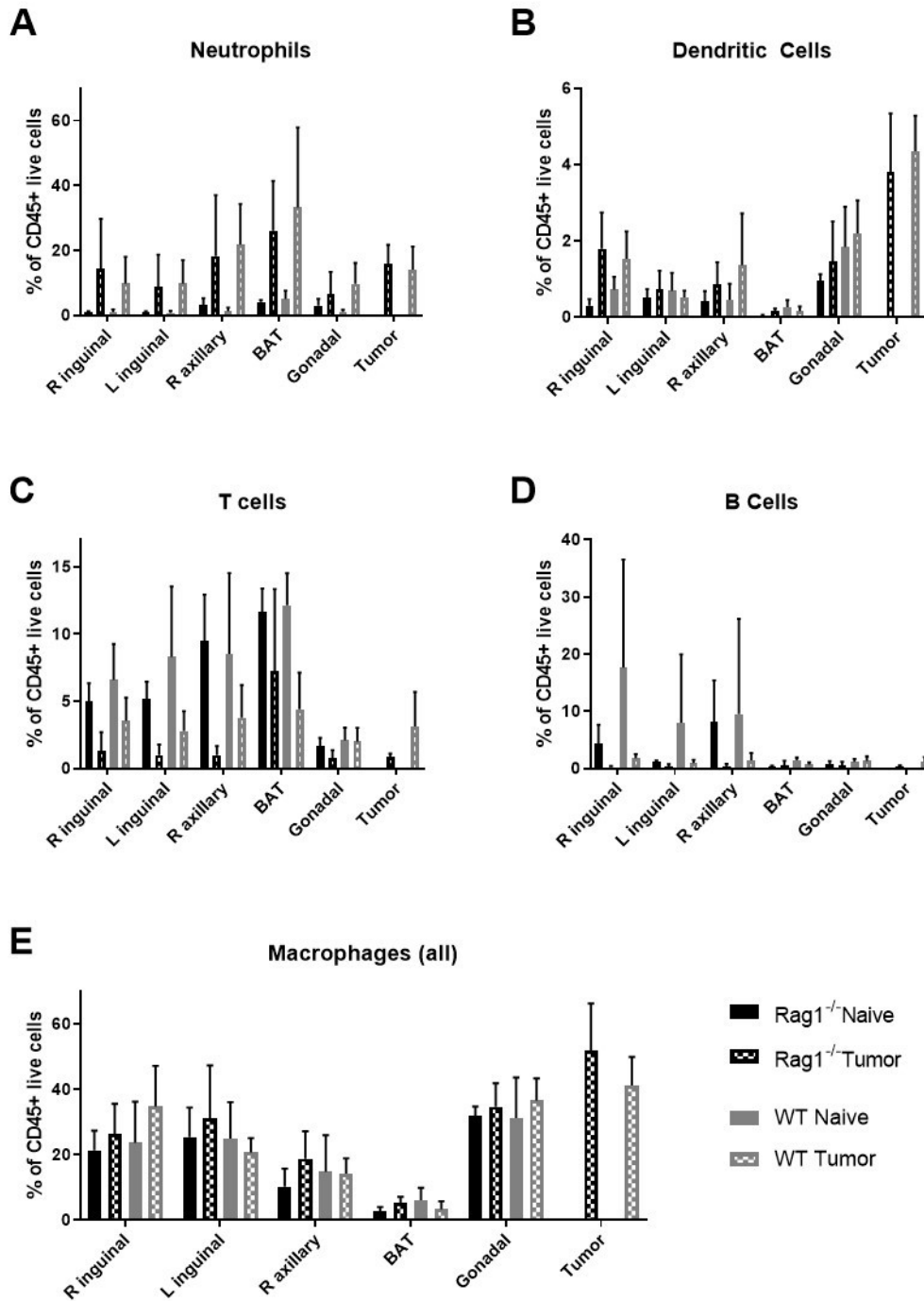


Figure 54. Various immune cell population percentages of CD45+ live cells from 4T1 allograft studies in wild-type and Rag1^{-/-} mice

Wild-type ('WT' in image) and Rag1^{-/-} mice of both male and female sex were inoculated with 4T1 cells or injected with control PBS and euthanized after tumor growth as described in methods. Flow cytometry results for (A) Neutrophils, (B) Dendritic cells, (C) T cells, (D) B cells, (E) Pan-macrophages. For all groups, n=4-5 mice.

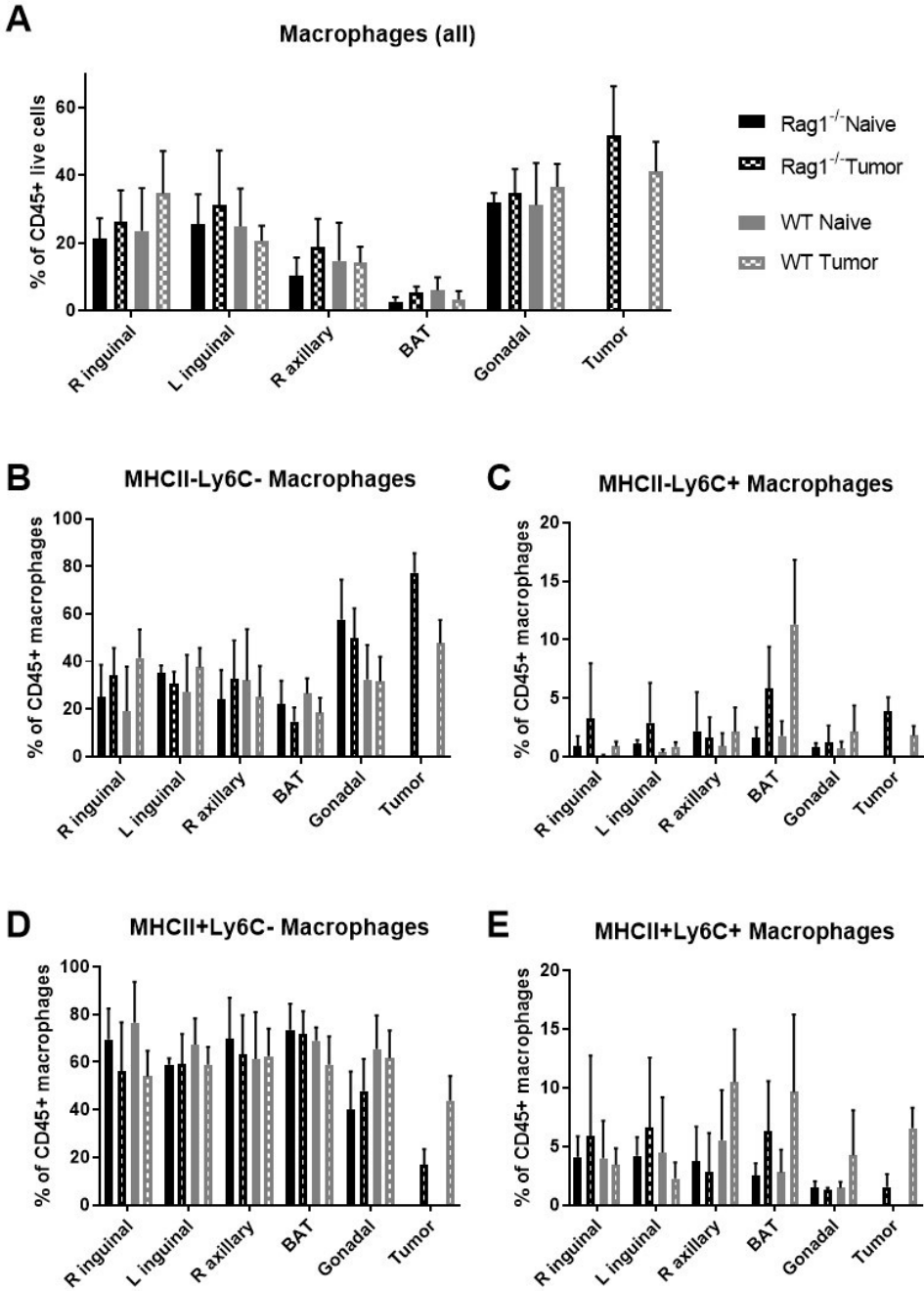


Figure 55. Macrophage population percentages of CD45⁺ live cells from 4T1 allograft studies in wild-type and Rag1^{-/-} mice

Wild-type ('WT' in image) and Rag1^{-/-} mice of both male and female sex were inoculated with 4T1 cells or injected with control PBS and euthanized after tumor growth as described in methods. Flow cytometry results for (A) Pan-macrophages, (B) MHCII⁺/Ly6C⁻, (C) MHCII⁺/Ly6C⁺, (D) MHCII⁺/Ly6C⁻, (E) MHCII⁺/Ly6C⁺. For all groups, n=4-5 mice.

5.3 Discussion

Since our results from Chapter 4 demonstrate that cancer cells induce changes to white adipocytes but do not cause WAT browning, we wanted to explore other possible mediators of these WAT browning changes demonstrated in Chapter 3. In their studies of a murine model of cachectic colon cancer, Petruzzelli and colleagues concluded that tumor-secreted IL6 is a direct driver of WAT browning [112]. We first investigated whether E0771, 4T1, and LLC cells secrete IL6 into the conditioned medium. All cells were treated with PMA and ionomycin, which overstimulate cytokine production, as well as brefeldin A and monensin, which act to inhibit intracellular protein and trap proteins in the endoplasmic reticulum. This method allowed us to make comparisons in *maximum* IL6 production of each cell type.

Compared with stimulated murine peritoneal cavity cells which contain inflammatory cells, E0771 cells produce significantly less IL6 when measured by intracellular flow cytometry (Figure 45A-B) and express over 100-fold less *Il6* mRNA (Figure 45C). When we tested IL6 expression in other cancer cell lines, we found that 4T1 and LLC cells both secrete more IL6 than E0771 cells, although still less than stimulated murine peritoneal cavity cells (Figure 46A), while mRNA expression of *Il6* was significantly decreased in LLC cells (Figure 46B). The expression and secretion of IL6 directly by breast cancer cells is variable, depending heavily on breast cancer cell type [226, 227]. Moreover, it is well established that IL6 is physiologically produced by non-adipocyte cells within adipose tissues [228], and can be modulated by breast adipose tissue in models of breast cancer [229]. Based on this information, and since tumor-derived IL6 has been directly implicated in other models of cancer-related WAT

browning [112], we asked whether it possesses the ability of generating white to brown transdifferentiation in our primary adipocyte cell culture system, regardless of its cell origin.

Since IL6 may come from a variety of cell types within the tumor microenvironment *in vivo*, we investigated whether our mature adipocytes *in vitro* have the necessary receptor machinery to ensure IL6-mediated signal transduction could occur. IL6 binds to either membrane-bound or soluble interleukin 6 receptor alpha (IL6RA, also referred to as CD126), and this IL6:IL6RA complex then associates with membrane-bound interleukin 6 signal transducer IL6ST (IL6ST, also referred to as GP130 or CD130) to induce signal transduction via the JAK/STAT3 pathway [230]. IL6ST expression is virtually ubiquitous, while expression of membrane-bound IL6RA is more cell-specific [231]. We confirmed that IL6ST is expressed in our mature, differentiated adipocytes, both on the membrane surface as measured by flow cytometry and by transcript levels with qPCR (Figure 47A-B). Interestingly, flow cytometry data for IL6RA was less than the isotype control, suggesting lack of, or minimal, receptor expression (Figure 47C). Our expression levels of *IL6ra* mRNA are consistent with this finding; while mRNA was detectable, it was 64- to 128-fold lower than stimulated peritoneal cavity cells (Figure 47D). Since soluble IL6RA is produced by breast cancer cells [232] and by monocytes and macrophages [231], these results prompted the inclusion of soluble IL6RA in our IL6 exposure experiments to mimic the tumor microenvironment and to ensure that signal transduction could occur.

With this information, we investigated whether exogenous IL6 exposure, with or without supplemented soluble IL6RA, would induce gene expression changes indicative

of WAT browning *in vitro*. We observed an increase in *Socs3* (suppressor of cytokine signaling 3) gene expression (Figure 48A) and STAT3 protein phosphorylation at the tyrosine 705 residue (Figure 48B) following just 30 minutes of exposure to both IL6 and IL6+IL6RA, not surprisingly more robust in the IL6+IL6RA group.

The suppressors of cytokine signaling (SOCS) family are molecules that inhibit JAK and STAT activation, regulated by its own signaling pathway: when STAT3 signaling is increased, transcription of SOCS3 is induced to act as negative feedback on the signaling pathway [145]. We demonstrate substantially increased *Socs3* expression in both IL6 and IL6+IL6RA treatment groups, which indicates that the JAK-STAT pathways is activated by these two treatment groups. The addition of IL6RA to the IL6 treatment results in an even more robust increase in *Socs3* expression – over 1024-fold that of the white adipocyte control – which tells us that IL6RA supplementation is useful to maximize this this signaling pathway *in vitro*. The phosphorylation of tyrosine 705 is canonical, while phosphorylation of serine 727 is a noncanonical pathway implicated in several physiologic states [233]. Tyr705 is associated with JAK signaling, while Ser727 is associated with MAPK signaling [234]. We are not surprised that the tyrosine 705 residue is phosphorylated, as JAK/STAT3 signaling is an established pathway in adipocyte plasticity. The lack of significant increases in serine 727 residue phosphorylation in the CL 316,243, IL6, or IL6+IL6RA treatment groups suggests MAPK/STAT3 signaling is not putative for WAT browning. Taking our *Socs3* mRNA expression data and STAT3 phosphorylation data together, these results confirm the bioactivity of IL6 and IL6RA via the JAK/STAT3 signaling pathway in our experimental model.

To our surprise, exposure of white adipocytes to IL6 or IL6+IL6RA for three days did not increase *Ucp1* or *Cidea* mRNA expression but instead resulted in a downward trend in expression (Figure 49A-B), similar to our observations in cancer conditioned medium (Figure 34A-B, Figure 37A-B) and coculture experiments (Figure 36A-B, Figure 39A-B). The IL6+IL6RA treated group expressed significantly more *Tnfrsf9* mRNA compared to control white adipocytes but had no significant changes in *Cidea* or *Ppargc1a* expression (Figure 49B), similar to our coculture results. Together, these results suggest that IL6-induced changes to adipocytes *in vivo* may be dependent on soluble receptors from other cells and additional signaling within the tumor microenvironment. *Lep* mRNA expression was significantly decreased with IL6+IL6RA treatment, similar to our observations in cancer conditioned medium and coculture experiments, but no changes were observed for *Cfd* or *Fasn* (Figure 49C). No change in any of the measured lipolysis markers was observed in white adipocytes treated with IL6+IL6RA (Figure 49D).

These results, especially the lack of increased *Ucp1* expression, contrast the findings of previous studies that implicate IL6 as a direct causative agent of WAT browning [112]. We found this, especially the expression of IL6 by LLC cells, to be interesting, as LLC cell-derived parathyroid hormone related peptide (PTHrp), but not IL6, has been implicated as a direct mediator of WAT browning in cancer [113], yet we demonstrate in Chapter 4 that our LLC-conditioned medium, which presumably contains both PTHrP and, based on our results here, at least detectable amounts of IL6, does *not* induce white to beige transdifferentiation. However, it is important to recognize the differences in our current study and design limitations that may account for these

discrepancies. IL6's role in WAT browning was implicated in a model of colon cancer, which is inherently different than breast cancer and the specific cancer cell line used in our studies. Other molecules, such as PTHrp, have been implicated in different models of CAC [113], so it is possible that other pathways may be driving the process of WAT browning in breast cancer. Whether IL6 exerts its effects directly or indirectly is still contested within the literature [235], so it is entirely possible that IL6's role in WAT browning in breast cancer may be indirect. It would be interesting to test whether constitutively-active STAT3 would yield different results, as that could provide useful information regarding the time-dependent nature of this signaling pathway. Additionally, other cytokines related to IL6 signaling should be explored, such as oncostatin M which interacts with gp130 [236], to better understand these signaling pathways *in vivo*.

To begin testing what processes may be involved *in vivo*, we exposed mature white adipocytes from two SVF cell strains to different tumor lysates and tumor-isolated CD11b+ cells. Tumor lysates inherently contain a variety of cells, including the tumor cells themselves as well as stromal cells and immune cells. In breast cancer, tumor lysate is being investigated as a vaccine because it contains multiple tumor-associated antigens which can induce a variety of immune cell responses [237]. A host of immune cell populations have been implicated in adipose tissue plasticity (reviewed by [238]). CD11b is part of the complement receptor type 3 (CR3, or macrophage-1 antigen MAC1) molecule expressed by monocytes/macrophages, granulocytes, and natural killer cells [239], and is implicated in cancer [240-242]. It appears that tumor lysate may increase *Ucp1* mRNA expression regardless of whether it came from early or late stage MMTV tumors, PyMT allograft tumors, or E0771 allograft tumors, although these results

were extremely variable. Cd11b exposure may also induce *Ucp1* expression, but the range was not as large as that in the tumor lysate groups (Figure 50). *Ucp1* mRNA increased in beige vs white cultured adipocytes, although this varied from 32- to 64-fold more between the two SVF (and was only significantly different in one of the two strains, Figure 50A). Furthermore, the effect of CL 316,243 was extremely variable between these two SVF strains – in the second strain, CL 316,243 exposure for 3 days resulted in higher *Ucp1* expression than beige control (Figure 50D). It is highly likely that this variability accounted for, at least part, some of the differences we saw in our exposure groups' results.

To work towards identifying cell populations that are crucial for WAT browning in breast cancer, we used two different BALB/c mouse strains, wild type and *Rag1*^{-/-}, and injected 4T1 breast cancer cells in the right inguinal WAT pad to induce allograft tumor formation. When we isolated the WAT from the right inguinal fat pad where tumor cell were injected and probed for *Ucp1* mRNA expression, wild-type mice with 4T1 tumors expressed roughly 2-fold more *Ucp1* mRNA, 4-fold more *Cidea* mRNA, 3-fold more *Prdm16*, and 2-fold more *Ppargc1a* (Figure 51C, gray bars) – all markers for thermogenic adipose tissue activity – which suggests that WAT browning occurs in this model. In *Rag1*^{-/-} mice with 4T1 tumors, inguinal WAT did not have changes in *Ucp1* expression compared to naïve controls, but had increased *Cidea* and *Ptk2b* (protein-tyrosine kinase 2-beta) expression and decreased *Ppargc1a* expression (Figure 51C, black bars).

V(D)J recombination-activating protein 1 (RAG1) is essential for variable (V), diversity (D), and joining (J) recombination for immunoglobulins and T cell receptors

(TCRs) in developing T and B cells [243], and deficiency of RAG1 in mice results in lack of mature T *and* B cell populations [244]. Other immune cell populations such as macrophages, dendritic cells, and natural killer cells remain intact and functional, making this mouse model ideal for providing information on which cell types may or may not be involved in WAT browning in breast cancer. While wild type mice with tumor have increased expression of several thermogenic genes (*Ucp1*, *Cidea*, *Ppargc1a*, and *Prdm16*), the *Rag1*^{-/-} mice only had increased expression of *Cidea*. This suggests that B and T cell involvement, either directly or indirectly, may be necessary for fully functional transdifferentiation towards the thermogenic adipose tissue phenotype. *Ptk2b* was increased in the inguinal WAT of *Rag1*^{-/-} mice with tumor. PTK2B is a nonreceptor protein kinase involved in responses to stimuli in several organ systems and a host of metabolic processes, including cultured beige adipocyte differentiation in our lab's murine immortalized SVF cell culture model [183]. In whole-body mouse studies, PTK2B (also known as PYK2) was shown to be important for glucose and weight homeostasis, as whole-body knock-out resulted in exacerbated weight gain and glucose intolerance on high fat diet [245]. In breast cancer (and others), most literature refers to PYK2 as an oncogene expressed by breast cancer cells with a variety of essential processes related to the development and progression of tumors (reviewed extensively by [246]). While we did not measure protein levels of PTK2B or its phosphorylation, we hypothesize that the increased *Ptk2b* expression by adipocytes is a maladaptive response to the presence of tumor, which aids in the growth and progression of breast cancer. For all of these results, it is important to note that the small scale of this

preliminary experiment warrants experimental replication, with a larger sample size, to determine whether any of these changes are significantly meaningful.

We next used flow cytometry to isolate and quantify the different immune cell populations within the right inguinal WAT (where 4T1 tumors grew) as well as distal WAT and BAT depots. Cells which are positive for CD45, also known as leukocyte common antigen 45, are white blood cells, excluding red blood cells. Of live cells, there did not appear to be any significant changes in the percentage of leukocytes (CD45+ cells) between naïve mice and mice with tumor. In the *Rag1*^{-/-} mice, there appears to be a slight increase in the percentage of CD45+ cells in mice with tumor in the inguinal and axillary WAT, but with our small sample size and large variability, significance cannot be determined. Similar trends are observed when we quantify each cell type as a percentage of live cells (Figure 52 and Figure 53) or of CD45+ live cells (Figure 54 and Figure 55), so for our discussion, we focus on the CD45+ live results as the results are more physiologically relevant to our questions since CD45+ cells are white blood cells.

The percentage of neutrophils and dendritic cells increases in adipose tissue pads of mice with tumor from both genotypes. Neutrophils can exhibit pro- or anti-inflammatory characteristics in cancer in an environment-dependent manner (reviewed by [247]). Furthermore, a 2017 study using human breast cancer samples found that tumor-associated neutrophils are present in human breast cancer and are most commonly present in triple negative subtypes [248]. Dendritic cells are known to be present in the breast cancer microenvironment [249], although their function has been shown to be dependent on local factors and may be subtype-specific [250]. Taken

together, it is not surprising that there are more neutrophils and dendritic cells in adipose tissues in our breast cancer allograft model.

There is, as inherently expected with the *Rag1*^{-/-} mice, a decrease in the percentage of T and B cells in naïve *Rag1*^{-/-} mice compared to wild-type naïve mice. Interestingly in both groups, the percentage of T and B cells in adipose tissue of mice with tumor *decreased* in both mouse strains. This is opposite of what we observe in neutrophil and dendritic cells. Most studies demonstrate an increase in T cell (especially T regulatory cells) and B cell populations in breast cancer [251-254]. It is possible that the decreased population levels that we see in the adipose tissue is the result of migration into the tumors themselves, but this is speculative.

There were no noticeable changes in the percentage of macrophages in adipose tissue pads between naïve wild type and naïve *Rag1*^{-/-} mice, which again is expected since *Rag1*^{-/-} is related to *only* T and B cell maturation [244]. In several adipose tissue depots in both genotypes, there are more macrophages (pan-macrophages) in the mice with tumor compared to naïve controls. This trend is most notable in the right inguinal WAT, which is where 4T1 cells were injected. This suggests that the local tumor microenvironment elicits as more location-specific increase in macrophage infiltration, which adds to prior literature demonstrating other immune cell infiltration in cancer [255, 256].

Macrophages are often necessary for various aspects of tumor progression (recently reviewed by [257]) and are associated with poor prognosis in many cancers [258]. In general, macrophages often begin as inflammatory and later transition to a more anti-inflammatory role [259]. While the historical classification of macrophages as

M1 “inflammatory” and M2 “anti-inflammatory” has recently been challenged [260], prior studies demonstrate that macrophage phenotype can change in cancer. Considering that macrophages can be activated into various phenotypes [260], we looked at different sub-classes of macrophages based on MHCII and Ly6C expression in the adipose tissue pads.

While there are inconsistent trends in the percentage of MHCII⁺/Ly6C⁺ macrophages, there is a decrease in the percentage of MHCII⁺Ly6C⁻ macrophages, especially in right inguinal WAT. The percentage of both Ly6C-positive and -negative MHCII-negative macrophages was increased in the fat pads of mice with tumor. In a mouse model of hepatocellular carcinoma, Wang and colleagues demonstrate temporal changes in macrophage phenotype: macrophages expressing MHCII were found early in tumorigenesis, while macrophages lacking MHCII increased in number with tumor progression; furthermore, they demonstrated that the early, MHCII⁺ macrophages were associated with tumor progression [261]. In mice, Ly6C expression is representative of pro-inflammatory monocytes/macrophages (reviewed by [262]). Taken together with our results, we found a general *decrease* in MHCII-positive cells and *increase* in MHCII-negative cells. The increase in MHCII-negative cells was most pronounced with the Ly6C-negative subtype, as this cell population increased by about 10% in adipose tissue in mice with tumor across both phenotypes (Figure 55). Our data therefore suggest, like prior literature, a shift towards anti-inflammatory macrophages (M2, or M2-like) in breast cancer.

Recently, alternatively activated macrophages have been implicated in WAT browning in burns [263]. In a murine model of high fat diet-induced obesity, an

increased M2-macrophage population resulting from knock-down of receptor interacting protein 140 (RIP140) was found to induce WAT browning and protect mice from high fat diet-induced obesity and insulin resistance [264], and obesity-driven inflammation, which is mostly pro-inflammatory, has been shown to inhibit beige adipogenesis [265]. Although the mechanism remains controversial [266, 267], alterations in macrophage phenotype are clearly involved in the WAT browning process. Taken together with our results demonstrating an increase in anti-inflammatory macrophages in adipose tissues of mice with cancer, we believe that WAT browning in cancer is likely mediated by this cell population *in vivo*.

There are several shortcomings to these experiments that future work can address. Experimental replication with a larger sample size powered to detect significant changes will be necessary. While our results point towards macrophages as a key driver of WAT browning in breast cancer, we cannot rule out other immune cell populations, especially those we did not measure in these preliminary studies. Other populations, such as eosinophils and T cells, have been shown to be involved in WAT browning (reviewed by [238]), so additional work must be done to explore the role of these cell types, both individually and in unison, in WAT browning. Additionally, these mice were raised in the barrier facilities at VCU, which is a 'cleaner' vivarium environment; it would be interesting to repeat these experiments in mice housed outside of this barrier vivarium to provide a more realistic environment that humans experience daily. Lastly, as the cell type(s) involved in this process are identified, individual molecular drivers and their mechanisms must be elucidated for future translation to alleviating WAT browning and CAC in patients.

5.4 Conclusion

In these experiments, we investigated the role of several cancer cell lines, and IL6, in an *in vitro* model of WAT browning. Our results demonstrate that E0771, 4T1, and LLC cells secrete detectable IL6 *in vitro*, but not abundantly. We show that while IL6 treatment induce changes to white adipocyte gene expression patterns, WAT browning does not occur directly via IL6 signaling, pointing to other cell types and/or interactions with cells within the tumor microenvironment as the potential driver of these thermogenic changes. We show a potential change in *Ucp1* expression after white adipocyte exposure to breast tumor lysate and tumor-isolated CD11b+ cells, and then moved *in vivo* to explore specific cell populations that may be driving WAT browning. Our results support prior literature regarding immune cell population changes in cancer and demonstrate an increase in anti-inflammatory macrophages in adipose tissue of mice with tumor. Further investigations are needed to understand the mechanisms behind these changes, the mechanisms by which these immune cell population induce WAT browning in cancer, and to test the effects on whole body energy expenditure and the development and progression of cancer-associated cachexia.

Chapter 6: Human Subjects Research – Maladaptive Adipose Tissue Activity in Cancer

6.1 Rationale

In the last several chapters, we have demonstrated WAT browning and altered adipocyte gene expression in breast cancer and mechanistically determined that immune cell population changes may be the source of WAT browning in breast cancer using murine and human *in vitro* and *in vivo* techniques. While the exact mechanisms of WAT browning in breast cancer are not yet elucidated, we wanted to begin to explore the translational potential of this work. A recent 2018 study demonstrated that BAT activity is greater in patients with active cancer compared with BAT+ individuals without active cancer [268]. In another study, researchers analyzed 1740 patients with PET/CT scans and found that only 30 (1.72%) showed activated brown adipose tissue activity; however, of those 30 BAT+ scans, 21 of them (70%) were in patients with cancer, while the other 9 (30%) were in patients without malignancy. Using data collected regarding the neoplastic status of each cancer patient (N0, N1, N2, N3) with multiple linear regression analysis, the authors demonstrated a significant association between BAT metabolic activity and neoplastic status, irrespective of age, BMI, or gender [107]. In another study performed in France, researchers took 5 PET/CT images for each of 33 female patients with either stage II or IIIA breast cancer at baseline and then after 1st, 2nd, 3rd, and 6th courses of chemotherapy. Patients with more BAT uptake had lower BMI, which suggests a relationship between thermogenic adipose tissue and CAC [269].

At the Virginia Commonwealth University Health System, we have access to a large amount of patient clinical data, as well as several different techniques with which

to measure energy expenditure in humans (Figure 2). We first ask if, via chart review, we could identify patients with cancer within the VCUHS who have activated BAT on PET/CT scans. Next, using whole room indirect calorimeters (which our lab has recently established incredible temporal accuracy for use in metabolic studies [15]), we ask two main questions: 1) if increased thermogenic adipose tissue activity in cancer is associated with increased resting energy expenditure, and 2) if increased temperature can 'quench' maladaptive thermogenic adipose tissue activity and decrease energy expenditure. Please refer to methods section 2.18 and Appendices for all protocols and pertinent information.

6.2 Results

6.2.1 Preliminary data: the VCUHS has an identifiable population of patients with cancer who have BAT activity detectable on PET/CT

Within the queried 10 year timeframe in the Montage database and the search parameters described in methods section 2.18.1, there were 210 results for patients at least 40 years of age with BAT activity documented. However, there were several cases of non-cancer-related indications for PET/CT, and several cases of multiple scans for one patient. After these results were removed, we were left with 164 unique patients at least 40 years of age with a history of cancer and at least one PET/CT with BAT activity noted in the report.

Of these 164 unique patients, 122 were female (74.4%), and 42 (25.6%) were male (Figure 56A). When stratified by age range (starting at age 40 based on inclusion criteria), we found 44 (26.8%) patients were between 40 and 49 years of age, 61

(37.2%) between 50-59, 40 (24.4%) between 60-69, 12 (7.3%) between 70-79, 6 (3.7%) between 80-89, and 1 (0.6%) between 90-99 (Figure 56B).

We also wanted to identify trends in primary cancer diagnosis and presence of BAT activity in this patient population. Of the 164 unique cases, we found that the majority (41, or 25%) of patients had a primary diagnosis of lymphoma (Figure 57). The second most common primary malignancy was breast cancer (34, or 20.7%), and the third most common was lung cancer (25, or 15.2%). The rest of the patients had a variety of different primary malignancies, and only 2 individuals had an unknown primary cancer diagnosis based on preliminary chart review.

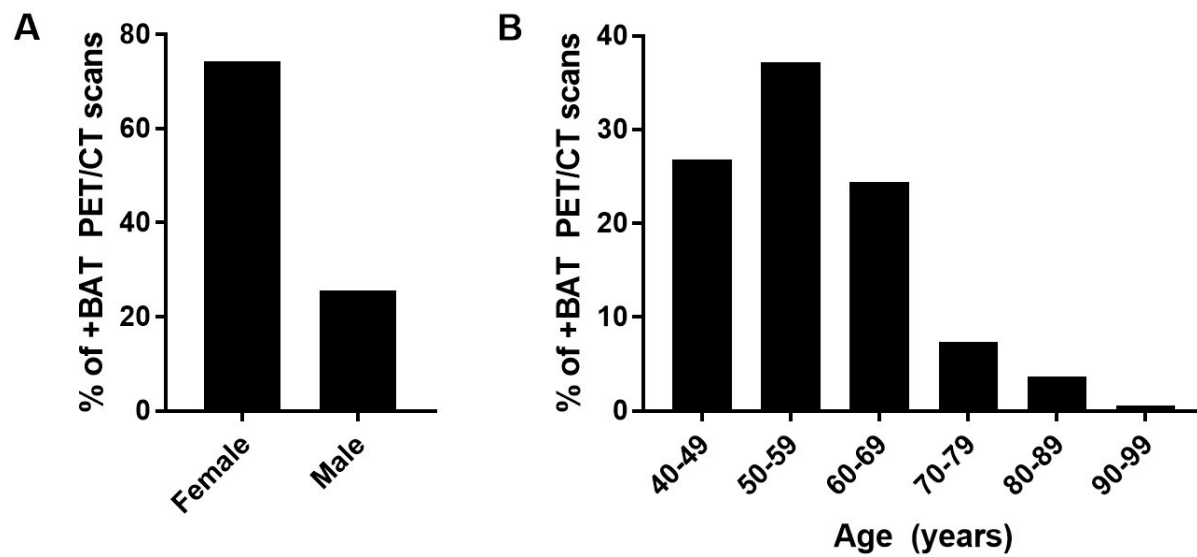


Figure 56. Gender and age in patients with cancer and BAT activity on PET/CT scans

A chart review was performed using the VCUHS medical record and medical imaging systems as described in methods. Percentage of patients with cancer and BAT activity on PET/CT scan by (A) gender and (B) age ranges.

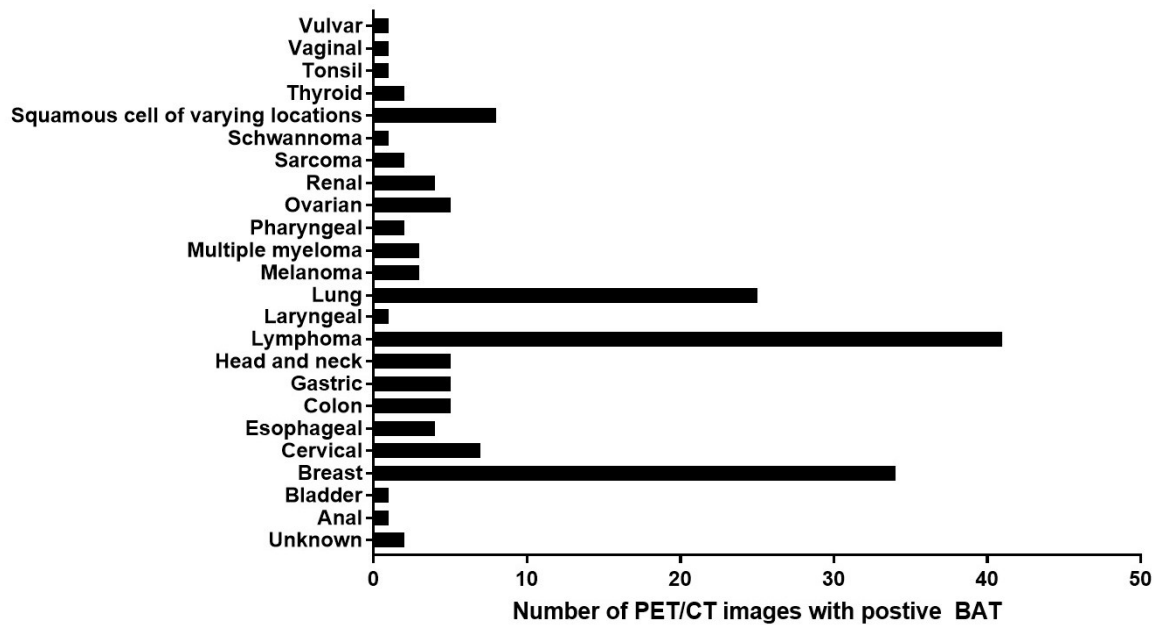


Figure 57. Primary cancer type in patients with cancer and BAT activity on PET/CT scans

A chart review was performed using the VCUHS medical record and medical imaging systems as described in methods. The y-axis includes all of the primary cancer types noted in patients with cancer and BAT activity on PET/CT scan, and x-axis is the number of patients with that particular cancer type.

6.2.2 Preliminary data: measurement of energy expenditure in human subjects with cancer

We began recruitment of participants for our study using whole-room indirect calorimetry as described in section 2.18.2. We just completed the first energy recording session for our first subject: a 54-year-old male with head & neck cancer who had BAT activity noted on the most recent PET/CT. The raw data are shown in Figure 58 and described in more detail in the figure legend. After analysis of the raw data, this subject's average respiratory exchange ratio was 0.73, and average metabolic rate was 0.88kcal/min (approximately 1267kcal/day).

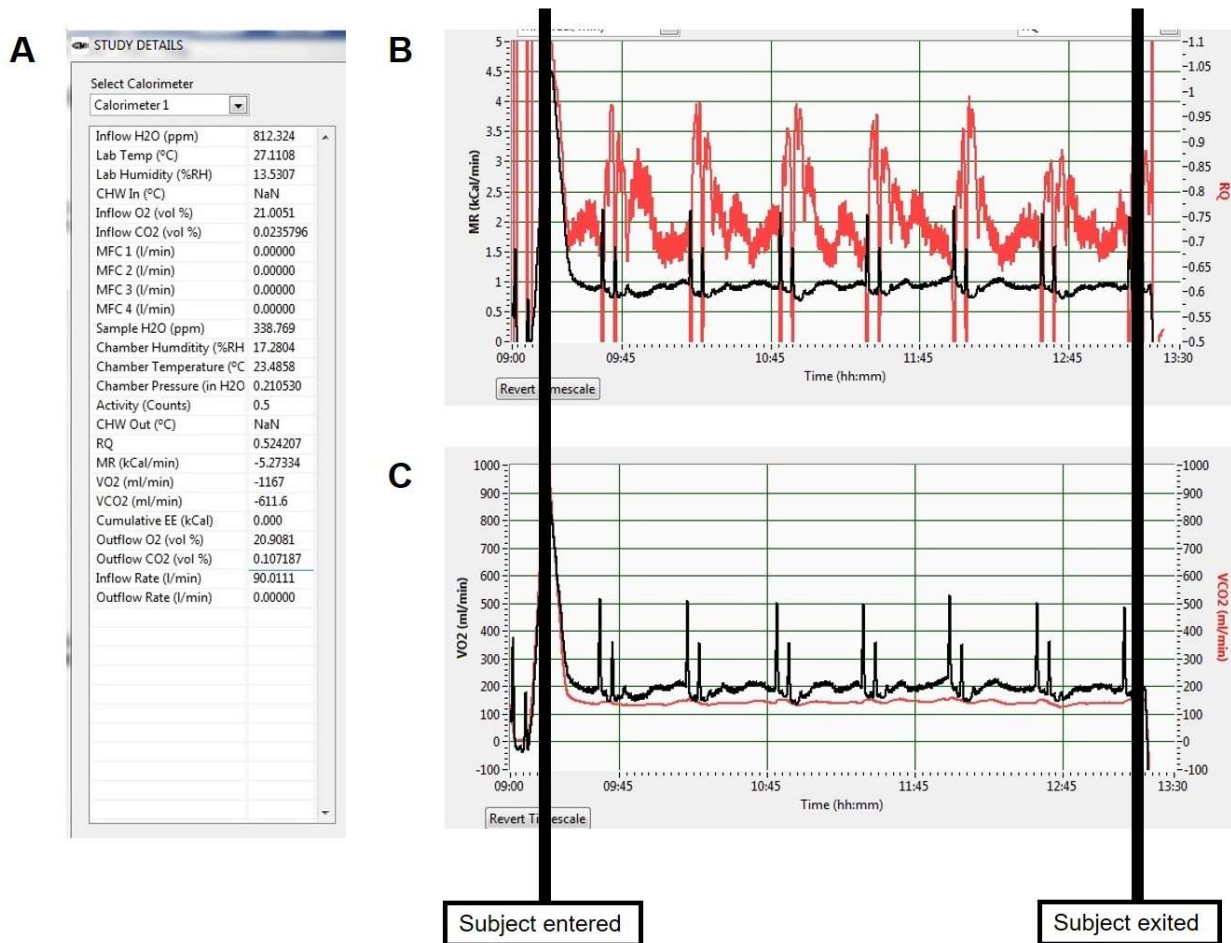


Figure 58. Whole-room indirect calorimetry, raw data

Raw data from energy recording using whole room indirect calorimeters from 54 year old male with head and neck cancer. (A) General information viewed during recording session to ensure calorimeters maintain appropriate air flow. (B) black line = metabolic rate (MR) in kcal/min; red line = respiratory exchange ratio (RER or RQ). (C) black line = volume of oxygen (VO₂) and red line = volume of carbon dioxide (VCO₂). Large vertical lines indicate time in which subject entered and exited the calorimeters.

6.3 Discussion

In the first part of this human subjects research, we used a retrospective approach to search the VCUHS records for our target patient population. We searched the past 10 years to gain a solid understanding of the prevalence of BAT activity at our institution. Our preliminary results demonstrate that there is indeed a targetable patient population at the VCUHS for studying the effects of thermogenic adipose tissue activity in patients with cancer. We found that the majority of these patients (74.4%) with cancer and BAT activity on PET/CT were female. This is not surprising, as several studies have demonstrated that women tend to have greater BAT mass and activity than men [38, 270, 271].

It is well established that thermogenic capacity decreases with age ([38], and recently reviewed by [272]); therefore, we chose 40 years as our minimum age as we believe it will optimize the number of patients with maladaptive thermogenic adipose tissue activity. We would expect very few patients over the age of 40 to have activated BAT activity on their PET/CT scans; however, we found 164 patients with what we hypothesize as *maladaptive* BAT activity. We were surprised that the number of patients in the 80-89 and 90-99 ages ranges with cancer and BAT activity were quite low, because although life expectancy at a given age decreases with age, the risk of dying of cancer has been shown to peak between ages 40-50 and *decrease* thereafter [273]. However, this could be due to a younger patient population seen at VCUHS versus other institutions in the surrounding area. We expected the 40-49 age range to contain the majority of BAT+ cases; however, we observed that most patients with cancer and BAT activity on PET were between 50 and 59 years of age. This adds stronger support

to our hypothesis that maladaptive activation of thermogenic adipose tissue occurs in cancer.

Our results demonstrate that lymphoma is the most common primary malignancy in this patient population. Of the 41 patients classified as having lymphoma as their primary malignancy, 13 had Hodgkin's lymphoma, 12 had non-Hodgkin's lymphoma, and 16 were not specified. Considering that interscapular BAT is in the same anatomical region as many lymph node changes, we are not certain that all of these patients have BAT activity versus cancerous FDG uptake. While CAC has been shown to affect patients across all cancer types [128], it has been previously reported that cancers such as gastric and esophageal have a higher prevalence of CAC compared to breast cancer (although we argue that the clinical implications of CAC in breast cancer are quite important, see Table 1). Interestingly, we found that the 2nd most common primary cancer in our patient population was breast cancer, with lung cancer being the 3rd most common. This suggests that maladaptive activation of thermogenic adipose tissue can certainly occur in breast cancer, strengthening the rationale behind our use of breast cancer as model for our *in vitro* molecular studies.

Several factors may have influenced our results. We must consider the potential for variability in training that may skew the prevalence of BAT activity towards lymphoma, breast, and lung cancers. As all three of these cancers are anatomically closer to interscapular region than other cancers such as colon cancer, it is possible the BAT activity may be under-represented in these results. Many factors may contribute to site-specific variations in patient population characteristics; regardless, it is clear that within the VCUHS, there are identifiable patients who have cancer with and without BAT

activity that we can recruit for energy characterization studies. We will continue to collect additional data on these patients as described in the methods, such as ethnicity, BMI, weight, diabetes status, and thyroid function testing. In the 2009 study demonstrating that approximately 25% of patients with breast cancer have cachexia, the authors did not provide additional subanalyses on sex or ethnicity in each cancer type [128]. While breast cancer is far more common in women than in men, it would be interesting to know whether any of the breast cancer patients in this study were men, and if so, if any of them had cachexia. Additionally, information regarding ethnicity would be extremely helpful to provide information regarding potential racial differences in breast cancer-related CAC. We hope that our data collection and analyses within our patient population will provide useful information in these areas. We know that thyroid function contributes to energy expenditure (reviewed in [274]), and BAT activity has been found to be more prevalent in lean individuals regardless of age [270]. It will be interesting to determine if this trend persists in this particular patient population as well.

The clinical implication of increased BAT activity relative to calorie utilization is not yet conclusive – estimates during cold exposure range from 15-25 kcal/day [108] to several hundred [275, 276]. While the prevalence of maladaptive thermogenic adipose tissue activity has been explored in several small studies as described in this chapter's rationale, the clinical relevance and implications of this increased activity is not well understood. We therefore developed a pilot study protocol using whole room indirectly calorimetry to determine whether BAT activity increases REE in human subjects with cancer at our institution. Our preliminary results from our first participant demonstrate our capacity to measure resting energy expenditure in patients with cancer using the

whole-room indirect calorimeters (Figure 58). Our first subject's average resting metabolic rate at 24°C was approximately 1267kcal/day. While this appears low when taken at face-value, it is crucial that this information be considered along with individual body weight at the time of study. We will be sure to calculate metabolic rate (kcal/day) per unit body weight (kg) for each subject to ensure appropriate comparisons in energy expenditure between the BAT-positive and BAT-negative groups.

We expect that patients with cancer with BAT activity on the PET/CT scans will have greater energy expenditure than those patients with cancer *without* BAT activity. Additionally, when we compare intra-individual energy expenditure differences, we expect that for patients with cancer who are BAT+, their resting energy expenditure will be decreased after exposure to the warmer temperature condition (27°C, or 80.6°F) compared to the 'room temperature' condition (24°C or 75.2°F). If the data support these hypotheses, our results would suggest that maladaptive browning does occur, and that warm temperature may have utility as a simple and safe therapeutic adjuvant.

Although we have recruited one participant, we are far from our goal of 36 patients. This highlights that clinical research and subject recruitment often takes longer than expected. Regardless, as we continue to recruit more subjects, we expect our results to provide a useful point-estimate difference in energy expenditure between BAT+ and BAT- patients with cancer, which is currently lacking in the literature. We have not analyzed the serum or urine samples obtained from this first patient, as we plan to analyze all samples together to minimize possible sources of result variability. However, we expect that our results, when combined with energy expenditure data, will provide useful clinical data for patients with cancer with or at risk for developing CAC.

Even once our recruitment is completed, it is important to note several limitations to this pilot study as we interpret our results and as subsequent studies are considered. First, we cannot say with certainty whether patients who had BAT activity on their PET/CT scan used for inclusion criteria continue to have measurable BAT activity by the time they are recruited into the study. BAT FDG uptake is not always consistent within a series of scans of a given patient [269, 277]. Ideally, we would have patients undergo repeat PET/CT before beginning any energy expenditure recordings. In this pilot study, we were financially limited and needed to utilize PET/CT scans that were already performed; however, in any larger studies that develop, funding should be requested to obtain these repeat PET/CT scans to confirm whether participants do or do not have activated BAT. Next, there is debate as to whether or not outdoor temperature and other related factors impact BAT activity on PET/CT scans, and if confers any change to energy expenditure; some studies demonstrate no significant relationship between BAT FDG uptake and outdoor temperature [269] while others demonstrate an association between photoperiod and seasonal variation with BAT activity [278]. We do our best to ensure patients complete their visits within the same season to decrease variability within a subject but individuals who enroll at different times of the year may have different results due to seasonal variability. It is also important to note that our study (and others) utilizing FDG uptake by BAT as a means to identify candidates inherently under-represents our ideal patient population: PET/CT is believed to underestimate the true prevalence of BAT activity (reviewed by [279]), and is not able to capture the more diffuse activity of beige adipose tissue activity in large WAT depots. Several methods are under development utilizing magnetic resonance or ultrasound (reviewed by [280]);

however, until these are validated and implemented in a widespread and cost-efficient manner, FDG PET/CT is still the 'gold standard,' even with its limitations.

6.4 Conclusion

In this Chapter, we utilized human subjects research to begin to understand the clinical and translational implications of maladaptive thermogenic adipose tissue activity in patients with cancer. Our results demonstrate that within the VCUHS, there exists a patient population comprised of individuals with cancer, at least 40 years of age, with brown adipose tissue activation. We argue that this BAT activity represents *maladaptive* adipocyte plasticity. Furthermore, though still in the early stages of recruitment and data collection, we demonstrate that our institution's whole-room indirect calorimeters are capable of measuring resting energy expenditure in patients with cancer. We expect to generate useful data quantifying the difference in energy expenditure between patients with cancer with and without BAT activity. Analysis of secondary endpoints will provide useful clinical data aimed towards improving quality of life in patients with cancer, especially those with or at risk for developing cancer-associated cachexia.

Chapter 7: Human Subjects Research – Unusual Case of Autoimmune Diabetes Mellitus in the Setting of Extra-Adrenal Paraganglioma with Loss of Succinate Dehydrogenase Expression

Please note that the majority of the material within this Chapter comes from our recently published manuscript, DOI 10.4158/ACCR-2018-0072. The final, definitive version of this paper has been published in AACE Clinical Case Reports: November/December 2018, Vol. 4, No. 6. [281]

7.1 Rationale

Even if cancer is not directly adjacent to adipose tissue, cancer can influence thermogenic adipose tissue activity as well as normal adipose tissue functions such as glycemic control. Recently, a case of hypermetabolic BAT was found in a patient with paraganglioma [282]. Paragangliomas are referred to as 'extra-adrenal pheochromocytomas,' originating from chromaffin cells in the sympathetic and/or parasympathetic ganglia [283]. Functional pheochromocytomas and paragangliomas are often associated with impaired glycemic control and exacerbation of pre-existing diabetes mellitus [284]. Here we present a unique case of autoimmune diabetes with glycemic control that follows the course of diagnosis and management for a norepinephrine-secreting paraganglioma.

7.2 Results (case report and bench laboratory findings)

A 55-year-old African American man with history of nephrolithiasis presented to an outside institution with flank pain and hypertension. Computerized tomography (CT) imaging with contrast revealed a heterogeneous retroperitoneal mass measuring 4.5 x

5.4 x 4.9 cm, located anterior to, and abutting, the inferior vena cava and abdominal aorta (Figure 59A). Further evaluation was consistent with paraganglioma: his plasma normetanephrines were 260 pg/mL (normal 0-145 pg/mL) and blood pressure was 160/90 mmHg (Figure 60). There is no known family history of paraganglioma, and only his mother has hypertension. He was started on Prazosin for control of hypertension in preparation for surgery.

Concurrently at the time of paraganglioma presentation and diagnosis, his blood glucose levels were elevated and anti-glutamic acid decarboxylase-65 (anti-GAD65) antibodies were 97.9 U/mL (normal 0-1.5 U/mL). He was therefore diagnosed with latent autoimmune diabetes of adults (LADA) and started a basal-bolus insulin regimen of Lispro 5U with meals and Detemir 25U at bedtime with prompt improvement in his hyperglycemia. Three months after initial diagnosis, the patient underwent a successful surgery, but review of the resected tumor by surgical pathology demonstrated the presence of positive margins. Additionally, there was involvement of an adjacent lymph node and foci of vascular space invasion. Immunostaining demonstrated loss of succinate dehydrogenase subunit B (SDHB) expression in the paraganglioma cells (Figure 61).

At post-surgery follow up, the patient reported normal blood pressure recordings and denied symptoms of catecholamine excess. He had self-decreased his insulin therapy because of reported improved glucose levels. Prandial insulin was discontinued.

Six months after surgery, the patient continued to report stable blood pressure without medication and no symptoms or signs of catecholamine excess. Point of care

HbA1c was 5.5% (37 mmol/mol) (normal < 5.6% or < 38 mmol/mol), and home measurements indicated post-prandial glucose levels ranging 120-140 mg/dL. Basal insulin was discontinued at this visit. Blood tests revealed normal catecholamine levels, but markedly elevated anti-GAD65 (above the assay's detection limit of 5000 U/mL) and anti-insulin antibodies of 137 μ U/mL (normal 0 μ U/mL) (Figure 60).

Eleven months after surgery, the patient was still normotensive and euglycemic without medication, with a point of care HbA1c of 5.7% (39 mmol/mol). However, CT imaging with contrast of the abdomen and pelvis showed a “small focus of ill-defined soft tissues” near the bifurcation of the abdominal aorta, soft tissue thickening at site of resected mass, and an enlarged left periaortic lymph node.

Eighteen months after surgery, the patient presented with unintentional weight loss, increased home glucose readings, and hypertension. Normetanephrines were significantly elevated at 841 pg/mL (normal 0-145 pg/mL), and HbA1c at 7.2% (55 mmol/mol). CT and subsequent 18 F-FDG positron emission tomography (PET)/CT imaging confirmed disease recurrence with metastasis to para-aortic lymph nodes (Figure 59B, C). Basal-bolus insulin regimen was resumed.

Two years after surgery, he has not experienced hypertensive crises but complains of fatigue and hyperhidrosis. Glycemic control had significantly worsened since the last visit, and his basal and bolus insulin doses were increased to Glargine 12U/day, and Lispro 5U before breakfast and 7U before lunch and dinner. He is also taking Benazepril 5mg and Hydrochlorothiazide 25mg daily for management of hypertension. He is undergoing cytoreductive therapy with Vincristine 1.4 mg/m², Dacarbazine 600 mg/m², and Cyclophosphamide 750 mg/m² (Figure 60).

Archival (formalin fixed, paraffin embedded) paraganglioma tissue obtained during the initial surgery was recut and mounted onto slides for IHC. Staining for GAD65 was negative in the paraganglioma (Figure 62).

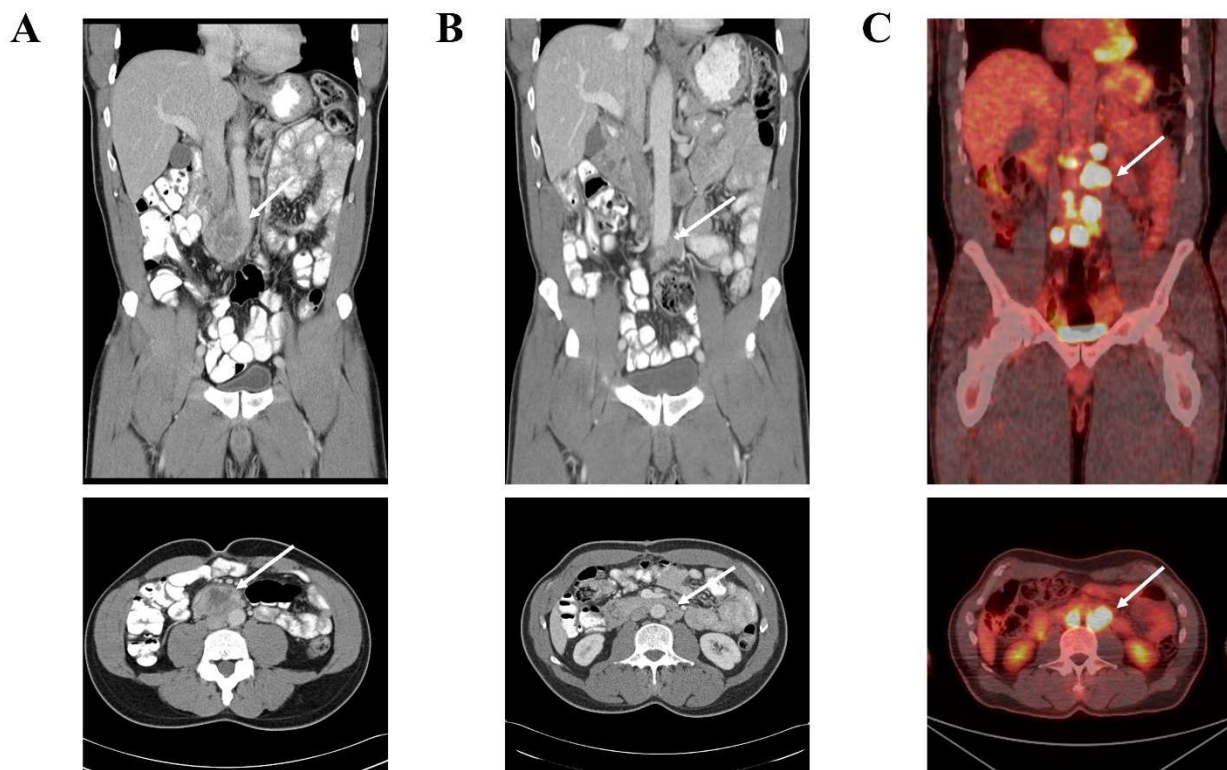


Figure 59. CT and PET/CT images at paraganglioma diagnosis and recurrence

Computed tomography images demonstrate the location of the paraganglioma at the time of first diagnosis (A) and at the time of recurrence (B). Recurrence was also imaged with positron emission tomography/computed tomography (C). White arrows point to the paraganglioma.

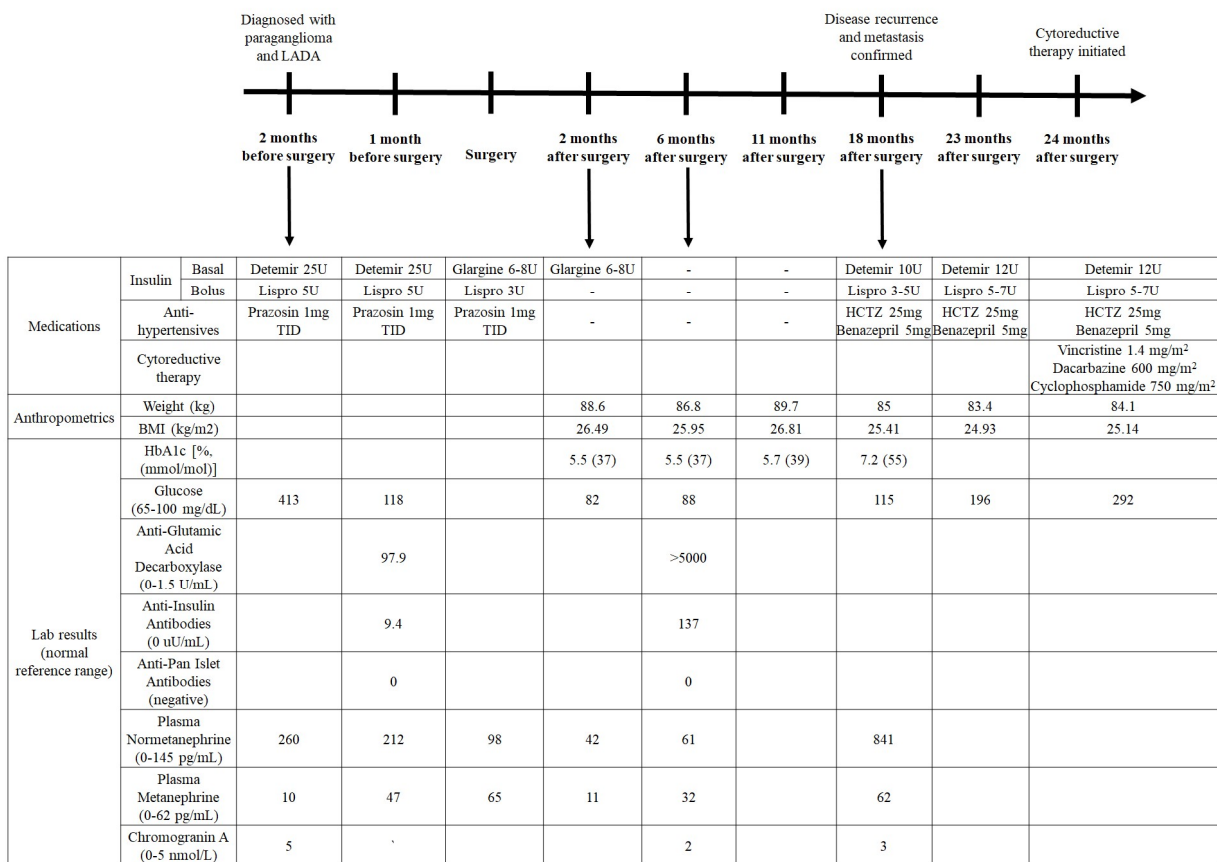


Figure 60. Timeline of events and corresponding lab results

The symbol “-” designates no medication at that time. Blank cells designate no value obtained at that time.

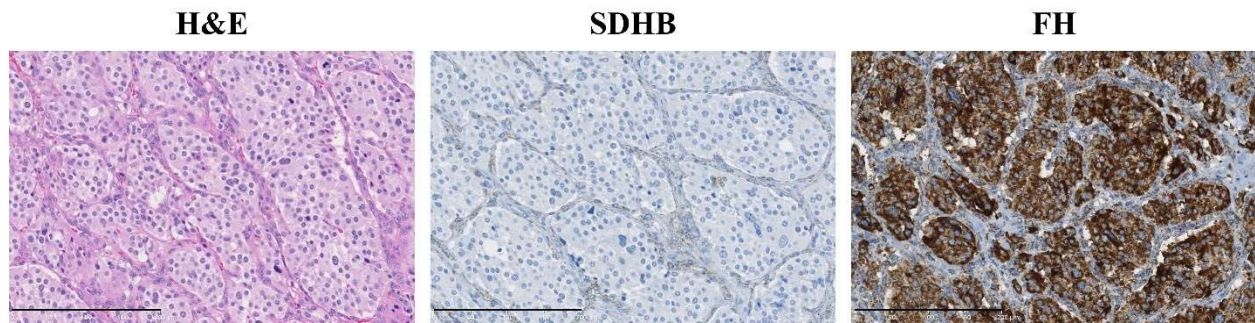


Figure 61. Loss of succinate dehydrogenase in paraganglioma

Immunohistochemistry imaging of the paraganglioma demonstrating loss of succinate dehydrogenase subunit B expression and positive fumarate hydratase staining for comparison. Scale bar in black is 200µm.

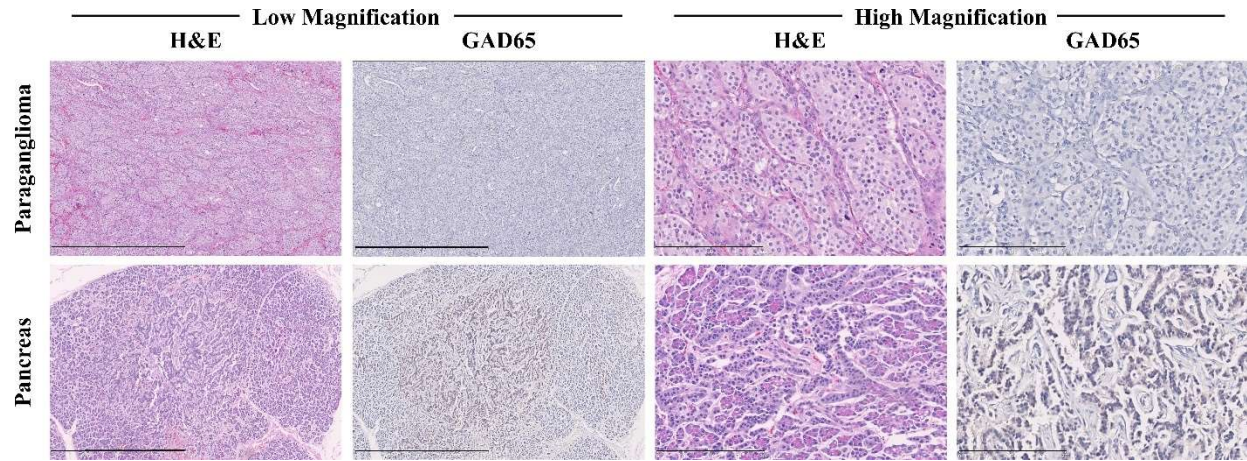


Figure 62. Lack of GAD65 expression in paraganglioma

Immunohistochemistry imaging of the paraganglioma (top) and control pancreas (bottom) with hematoxylin and eosin and glutamic acid decarboxylase-65 at low and high magnifications. Low magnification scale bar is 1 mm; high magnification scale bar is 200µm.

7.3 Discussion

Functional pheochromocytomas and paragangliomas are classified into three major phenotypes: adrenergic, noradrenergic, and dopaminergic [285]. Most functional paragangliomas are noradrenergic, whereas many pheochromocytomas can be adrenergic because of the presence of phenylethanolamine N-methyltransferase in the adrenal medulla [286]. Because of excess catecholamine secretion, functional pheochromocytomas and paragangliomas are often accompanied with hyperglycemia. The mechanisms by which catecholamines induce this glucose intolerance include insulin resistance [287] with decreased glucose uptake into muscle and adipose tissue [288], decreased insulin release [289, 290], and stimulation of glucagon secretion [291]. We could not address the possibility of germline *SDH* mutations [292] since the patient declined genetic testing; however, it is well-documented that *SDH* mutations in paragangliomas confer an aggressive tumor phenotype and increased metastatic potential [293-295], so the clinical course of this patient's tumor burden is not surprising. Cases have been presented demonstrating that in patients who already had type 2 diabetes, paraganglioma resection dramatically improved their glycemic control [296]. However, to the best of our knowledge, this is the first description of a new diagnosis of autoimmune diabetes in the setting of a paraganglioma. The patient's age at presentation, autoantibody positivity, and insulin dependence all meet the conventional requirements for the diagnosis of LADA [297]. It is interesting that the timing of autoimmune diabetes diagnosis matches that of the paraganglioma diagnosis, and that the waxing and waning of poor glycemic control seems to correlate with the timing of paraganglioma surgical debulking and subsequent recurrence. We postulate three

possible explanations: one, this patient has two concurrent, but unrelated, diagnoses of paraganglioma and LADA; two, the recurrence of poor glycemic control is caused primarily by the norepinephrine secreted by the paraganglioma; and three, this patient's paraganglioma elicits an autoimmune response that targets the pancreatic beta cells leading to autoimmune diabetes.

We cannot rule out the possibility that this patient's glycemic trends are the result of the 'honeymoon phase,' or period of 'remission,' that can occur in patients with type 1 diabetes, during which patients are often able to halt most or all of their insulin therapy and maintain euglycemia [298]. In the pediatric population, this period of remission can vary between three months to 13 years (reviewed by [298]). In adults, some cases report remission lasting beyond 14 months after cessation of insulin regimens [299], and this patient's period of euglycemia without insulin fits within that timeframe.

Closely related to the first possible explanation, we do not expect this patient's glucose intolerance to be solely attributable to norepinephrine secretion by the paraganglioma. Norepinephrine excess can contribute to hyperglycemia, but less significantly than epinephrine because of differences in receptor affinities [296]. Additionally, the patient's anti-GAD65 antibodies were elevated at the time of paraganglioma diagnosis, and then were substantially increased at the time of confirmation of disease recurrence. The patient had no neuromuscular symptoms or signs that could be associated with Stiff-person syndrome [300]. The presence of anti-GAD antibodies is clearly indicative of autoimmune-related diabetes, which to the best of our knowledge, is not attributable to norepinephrine excess.

Lastly, while we do not have anti-GAD65 levels preceding the diagnosis, or during the period of euglycemia post-surgery, their trend is suggestive of an autoimmune process which would result in loss of beta cell function, a condition which has not been described before in the setting of a paraganglioma. The patient's improvement in glycemic control after surgical debulking could be the result of removal of antigen within the paraganglioma, whose burden increased as the tumor recurred and metastasized. Although our IHC results did not show GAD65 antigen present within the paraganglioma tissue, which supports one of the first two proposed hypotheses, we cannot exclude a tumor-induced autoimmune process toward other epitopes not recognized by the antibody utilized in our IHC. Interestingly, GAD65 expression in a small cell lung cancer has been reported in a case of LADA with high anti-GAD65 titers, indicative of a paraneoplastic syndrome [301]. The extraordinary rise in plasma antibody titer concomitant with the relapse of tumor is consistent with an increase in exposure to the antigen. This would support the hypothesis of a novel form of paraneoplastic autoimmune diabetes in the setting of paragangliomas, and should be further evaluated in future work.

While not directly adjacent to adipose tissue, these results clearly demonstrate the role of tumor and tumor microenvironment on whole-body energy and glucose homeostasis, which inherently includes adipose tissue.

7.4 Conclusion

Catecholamine excess can cause hyperglycemia in patients with functional paragangliomas. This is a unique case of autoimmune diabetes diagnosed in the setting

of a functional paraganglioma, with glycemic control closely related to paraganglioma disease burden. Further research and testing should be done in patients with pheochromocytomas and paragangliomas to investigate a possible autoimmune role in certain cases. Additionally, this work highlights the importance of understanding the implications of cancer on whole-body energy and glucose homeostasis.

Summary

The role of adipose tissue in CAC is complex. Our results show that adipose tissue plasticity occurs in breast cancer. First, we demonstrate in murine models of breast cancer as well as in human samples of breast cancer that white adipose tissue, in the presence of tumor, undergoes WAT browning to a beige adipose tissue phenotype. We show that this effect is localized and seems to occur earlier in cancer progression (Figure 63A).

Next, we moved into *in vitro* models of adipogenesis and cancer to investigate the role of cancer secreted factors, as well as cancer:adipocyte cross-talk, on adipocyte plasticity. We show in both human and mice cell culture that cancer secreted factors and cross-talk decrease mRNA expression of classic WAT-related genes. In mice, we show that cross-talk specifically results in an increase in lipolysis-related mRNA expression, and in humans, cancer secreted factors reduce white adipocyte lipid droplet size. Regarding WAT browning, our results strongly suggest that in mice, neither cancer secreted factors nor cross talk with adipocytes is able to induce WAT browning; however, in human cell culture, our results are less conclusive (Figure 63B).

We demonstrate that IL6+IL6RA, regardless of its source, is able to induce IL6-mediated signaling through STAT3 phosphorylation; however, that signaling alone is not sufficient to directly induce WAT browning. The last part of our bench laboratory experiments, we present preliminary data suggesting that immune cell population shifts within the white adipose tissue of mice with breast cancer tumors may be source of WAT browning (Figure 63B).

Lastly, we show that the VCUHS has an identifiable population of patients with cancer over the age of 40 with brown adipose tissue activity on PET/CT scan. Our pilot clinical study is currently underway to test our hypothesis that this increased brown adipose tissue activity is *maladaptive* in this patient population, and to show the effects of these changes on whole body energy expenditure as well as other relevant secondary endpoints.

In conclusion, we have demonstrated that adipose tissue plasticity occurs in breast cancer and certain components are caused by different drivers with the tumor microenvironment. We predict that further exploration of the exact mechanisms and translational implications will provide useful information to lead to new therapeutic treatments for patients with cancer-associated cachexia.

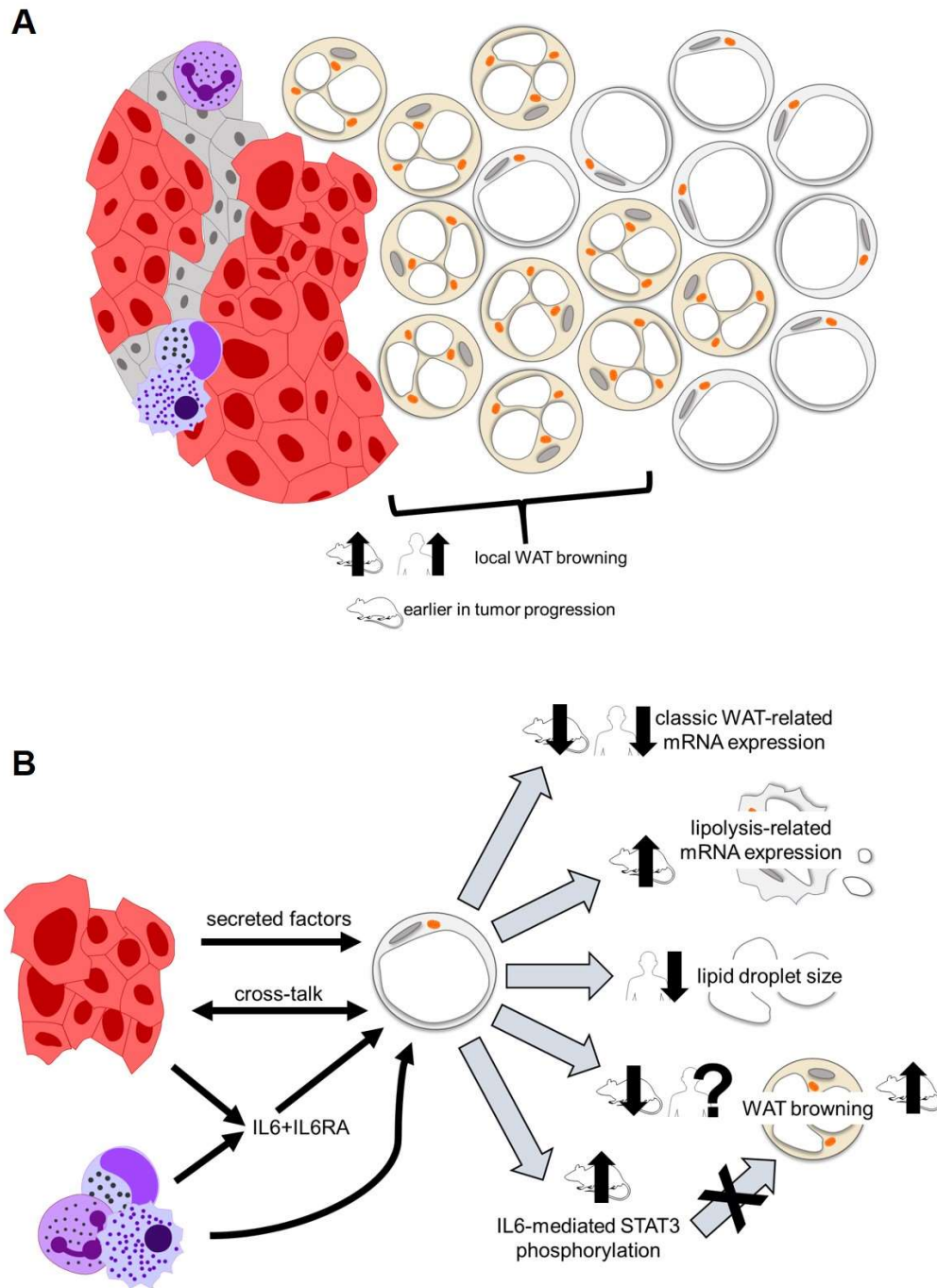


Figure 63. Summary of work

(A) Local WAT browning occurs in murine and human breast cancer, and in murine models we demonstrate that this occurs earlier in tumor progression. (B) Using *in vitro* cell culture models, we demonstrate that cancer secreted factors, cross talk between cancer cells and adipocytes, IL6+IL6RA, and immune cell population shifts are capable of inducing as host of effects related to adipocyte plasticity.

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Vita

Janina Vaitkus Pearce was born August 31st, 1991 as Janina Aldona Vaitkus in Montgomery County, Maryland, and is an American Citizen. She graduated from South River High School in Edgewater, Maryland, in 2009. She received her Bachelor of Science in Bioengineering, Cum Laude, from the University of Maryland College Park in Spring 2013. In Summer/Fall 2013, she entered the M.D.-Ph.D. graduate program at Virginia Commonwealth University School of Medicine.

Presentations from or related to this work

Please note my maiden name (Janina Aldona Vaitkus, or Vaitkus, J. A.) or my married name (Janina Vaitkus Pearce, or Pearce, J. V.) for these conferences and presentations.

Submitted: S Chen, J Luo, L Zhao, **JV Pearce**, FS Celi. Fast Detection of Brown Adipose Tissue in Cancer Patients by Localizing Supraclavicular Fossae. SNMMI Annual Meeting; June 22-25 2019; Anaheim, California, USA.

J.A. Vaitkus, J.S. Farrar, B. Ni, F.S. Celi. WHITE ADIPOSE TISSUE BROWNING IN BREAST CANCER IS NOT DIRECTLY MEDIATED BY TUMOR CELL SECRETOME OR INTERLEUKIN-6. Virginia Commonwealth University Massey Cancer Center 2018 Cancer Research Retreat, June 8, 2018; Richmond, Virginia, USA.

J. Vaitkus, S. C. Smith, and F. S. Celi. UNUSAL CASE OF AUTOIMMUNE DIABETES IN THE SETTING OF EXTRA-ADRENAL PARAGANGLIOMA WITH SUCCINATE DEHYDROGENASE DEFICIENCY. 27th Annual AACE Scientific & Clinical Congress; May 17, 2018; Boston, Massachusetts, USA.

P. Rao, B. Ni, **J. Vaitkus**, P. Puri, F. Celi. THYROID HORMONE HOMEOSTASIS ACROSS THE SPECTRUM OF FATTY LIVER DISEASE. 27th Annual AACE Scientific & Clinical Congress; May 19, 2018; Boston, Massachusetts, USA.

J. Vaitkus, B. Ni, and F. S. Celi. Local white adipose tissue browning in breast cancer is not mediated by direct tumor-adipocyte interactions. American Physician Scientist Association Annual Meeting, April 20, 2018, Chicago Illinois USA.

JS Farrar, **J.A. Vaitkus**, B. Ni, FS Celi. Quantitative real-time PCR for the assessment of white, classical brown, and beige adipose tissue. Keystone Symposia Obesity and Adipose Tissue Biology; January 23, 2017, 2017; Keystone, Colorado USA.

J.A. Vaitkus, J.S. Farrar, B. Ni, F.S. Celi. WHITE ADIPOSE TISSUE BROWNING IN BREAST CANCER. Virginia Commonwealth University Massey Cancer Center 2016 Cancer Research Retreat, June 17, 2016; Richmond, Virginia, USA.

Publications from or related to this work

Please note my maiden name (Janina Aldona Vaitkus, or Vaitkus, J. A.) or my married name (Janina Vaitkus Pearce, or Pearce, J. V.) for these publications.

*Submitted: **Pearce JV**, Farrar JS, Lownik JC, Ni B, Chen S, Kan TW, Celi FS. E0771 breast cancer cells and interleukin 6 alter gene expression patterns but do not induce browning in cultured white adipocytes. Biochemistry and Biophysics Reports.*

Vaitkus JA, Smith SC, Boikos SA, Celi FS. Unusual Case of Autoimmune Diabetes Mellitus in the Setting of Extra-Adrenal Paraganglioma with Loss of Succinate Dehydrogenase Expression. AACE Clinical Case Reports: November/December 2018, Vol. 4, No. 6, pp. e457-e462

Vaitkus JA, Celi FS. The role of adipose tissue in cancer-associated cachexia. Exp Biol Med (Maywood). 2017;242(5):473-481.

Ni B., Farrar JS, **Vaitkus JA**, and Celi FS. Metabolic Effects of FGF-21: Thermoregulation and Beyond. Front Endocrinol (Lausanne), 2015. 6: 148.

Vaitkus JA, Farrar JS, and Celi FS. Thyroid Hormone Mediated Modulation of Energy Expenditure. Int J Mol Sci, 2015. 16(7): p. 16158-75.


Appendices: Human Subjects Research

All documents current as of 2/4/2019

Appendix A

Human Subjects Research IRB Study Smartform

1/7/2019 Print: HM20009089 - Malesadaptive adipose tissue activity in cancer



Date: Monday, January 07, 2019 8:42:48 AM
ID: HM20009089

View: SF - Study Identification

Print Close

HM20009089 - Francesco Celli
Malesadaptive adipose tissue activity in cancer

Study Identification

- * Select the Principal Investigator:**
Francesco Celli
- * Study Title:**
Malesadaptive adipose tissue activity in cancer
- * Is this a student or trainee project in which activities will be carried out by that individual under your supervision:**
☒ Yes
☐ No
- Select any associated VCU IRB protocols:**
ID PI
There are no items to display
- Select all individuals who are permitted to edit the IRB protocol and should be copied on communications (study staff will be entered later). These individuals will be referred to as protocol editors:**

Last Name	First Name	E-Mail	Phone	Mobile
Charron	Martin	mcharron@vcu.edu		
Chen	Shanahan	schen3@vcu.edu		
Del Fabbro	Egidio	edelfabbro@vcu.edu	8048289909	
Gel	Tamas	tgel@vcu.edu	8046262123	
Pearce	Janina	valtkus@vcu.edu	0000000000	
Stamatoull	Angeliki	amstamatoull@vcu.edu		

ID: HM20009089 View: SF - Student/Trainee Investigator Contact

Student/Trainee Investigator Contact

If this project involves more than one student / trainee investigator, identify the primary contact here and list all student / trainee investigators in the Personnel section.

Also ensure all are listed as protocol editors if they need to be copied on IRB correspondence and have authority to make edits.

- * Name:**
Janina Valtkus Pearce
- * VCU Email:**
valtkus@vcu.edu
- * Phone:**
4437583785

ID: HM20009089 View: SF - Research Determination

Research Determination

- * Select one of the following that applies to the project:**
☒ Research Project or Clinical Investigation
☐ Exception from Informed Consent for Planned Emergency Research
☐ Humanitarian Use of Device for Treatment or Diagnosis

<https://irb.research.vcu.edu/irb/Resources/Administration/Project/PrintSmartForms?Project=com.webbridge.entity.Entity%5B0ID%5B8EAE13E7697DB9F...> 1/38

- ☐ Humanitarian Use of Device for Clinical Investigation
- ☐ Emergency Use of Investigational Drug, Biologic or Device
- ☐ Treatment Use (Expanded Access to Investigational Product for Treatment Use)
- ☐ Center or Institute Administrative Grant Review

2. * VCU requires that all clinical research studies be evaluated to determine if a Coverage Analysis is required. Has your study been evaluated by an Institutionally designated Coverage Analysis Specialist:

- ☒ Yes
- ☐ No
- ☐ Not Applicable

***If No above, please contact your school or center research administration office for the support of an Institutionally-designated VCU Coverage Analysis Specialist.

***Not Applicable should only be checked if this study is **not** Clinical Research.

ID: HM20009089

View: SF - Federal Regulations

Federal Regulations

1. * Is this a Clinical Trial? A clinical trial is a study that prospectively assigns human subject(s) to an Intervention(s) and evaluates the effects of the intervention on health-related outcomes:

- ☒ Yes
- ☐ No

2. * Is this a FDA regulated study:

- ☐ Yes
- ☒ No

3. * Is this study supported by the Department of Defense (DoD):

- ☐ Yes
- ☒ No

4. * Check if any of the following funding sources apply to this research (including Direct and/or Indirect funding):

None of the above

ID: HM20009089

View: SF - Personnel

Personnel

1. * Indicate all VCU/VCUHS personnel, including the PI, who will be engaged in this study:

Name	Roles	Roles - Other	Responsibilities	Responsibilities - Other	Qualifications	Qualifications COI - Other	Investigator
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1/7/2019

Print: HM20009089 - Maladaptive adipose tissue activity in cancer

	Name	Roles	Roles - Other	Responsibilities	Responsibilities - Other	Qualifications	Qualifications - Other	COI Investigator
View	Francesco Celi	Principal Investigator		Data Analysis Project Coordination Data Collection - Direct Observation Participant Consent Data Collection - Lab Data Management Data Collection - Clinical Participant Identification Data Entry Study Design Data Coding Participant Recruitment Data Collection - Interviews/Surveys		Experience - Research Experience - Related Skills Experience - Clinical Education and/or Professional Preparation		yes
View	Janina Pearce	Trainee/Student		Data Analysis Project Coordination Data Collection - Direct Observation Participant Consent Data Collection - Lab Data Management Data Collection - Clinical Participant Identification Data Entry Study Design Data Coding Participant Recruitment Data Collection - Interviews/Surveys		Student		yes
View	Egidio Del Fabbro	Co/Sub-Investigator		Data Analysis Project Coordination Data Collection - Direct Observation Participant Consent Data Collection - Lab Data Management Data Collection - Clinical Participant Identification Data Entry Study Design Data Coding Participant Recruitment Data Collection - Interviews/Surveys		Experience - Research Experience - Related Skills Experience - Clinical Education and/or Professional Preparation		yes

<https://irb.research.vcu.edu/irb/ResourceAdministration/Project/PrintSmartForms?Project=com.webbridge.entity.Entity%5B0ID%5BEAE13E7697DB9F...> 3/38

1/7/2019

Print: HM20009089 - Maladaptive adipose tissue activity in cancer

	Name	Roles	Roles - Other	Responsibilities	Responsibilities - Other	Qualifications	Qualifications - Other	COI Investigator
View	Shanshan Chen	Co/Sub-Investigator Statistician		Data Analysis Project Coordination Data Collection - Direct Observation Participant Consent Data Collection - Lab Data Management Data Collection - Clinical Participant Identification Data Entry Study Design Data Coding Participant Recruitment Data Collection - Interviews/Surveys		Experience - Research Experience - Related Skills Experience - Clinical Education and/or Professional Preparation		yes
View	Tamas Gal	Consultant Statistician		Data Analysis Data Management Data Collection - Clinical Data Entry Data Coding		Experience - Research Experience - Related Skills Experience - Clinical Education and/or Professional Preparation		yes
View	Martin Charron	Consultant		Data Analysis Data Management Data Collection - Clinical Participant Identification Data Entry Data Coding		Experience - Research Experience - Related Skills Experience - Clinical Education and/or Professional Preparation		yes
View	Angeliki Stamatouli	Co/Sub-Investigator		Data Analysis Project Coordination Data Collection - Direct Observation Participant Consent Data Collection - Lab Data Collection - Clinical Participant Identification Data Entry Participant Recruitment Intervention Services Clinical Services Data Collection - Interviews/Surveys		Experience - Research Experience - Clinical Education and/or Professional Preparation		yes

2. Identify all non-VCU personnel who will be engaged in this study AND who DO NOT have IRB approval for this study from their own Institution.

Name Roles Roles - Other Responsibilities Responsibilities - Other Qualifications Qualifications - Other COI Investigator
There are no items to display

<https://irb.research.vcu.edu/irb/ResourceAdministration/Project/PrintSmartForms?Project=com.webbridge.entity.Entity%5B0ID%5BEAE13E7697DB9F...> 4/38

3. CV/Biosketch: (required for PI, Medically/Psychologically Responsible Investigators and Student/Trainee Investigators)

ID: HM20009089

View: SF - Conflict of Interest

Conflict of Interest

1. * To the best of your knowledge, do you (as PI) or any other engaged individual hold a financial conflict of interest related to this study?

- ☐ Yes
- ☒ No

2. If Yes, provide:

- Name(s) of the engaged conflicted individual(s)
- Brief description of the financial conflict of interests

3. * Describe any potential non-financial conflicts of interest for members of the research team that could impact the conduct of the study (if None, please state "None"):
None.**4. Describe any institutional conflict of interest with this research that you or any member of the research team may be aware of:**

ID: HM20009089

View: SF - Communication Plan for Research Team

Communication Plan for Research Team

1. * Describe the process that will be used to ensure that all persons at all involved sites assisting with the research are adequately informed about the protocol and their research related duties and functions:

For Aim 1 specifically, Radiology and Biostatistics staff will train investigators in how to search the nuclear medicine database and PET image results, using the Montage program and Cerner databases. For both Aims, everyone on the protocol has already completed Cerner training and has access to the Cerner database. All listed personnel on this protocol will have access to and will document searches and data collection, and will update the research team regularly. We will communicate regularly via email or in person as charts are being reviewed (Aim 1) and participants are recruited and data is collected (Aim 2). There will be a weekly, in-person meeting with Dr. Cell, Dr. Chen, and Janina to discuss progress and challenges. Update emails will be sent monthly to the whole team, and specific details can be discussed with team members in person as needed.

ID: HM20009089

View: SF - IRB Panel Setup

IRB Panel Setup

1. * To which IRB is this study being submitted for review:

- ☒ VCU IRB
- ☐ Western IRB
- ☐ NCI Central IRB
- ☐ Other IRB

2. If Other IRB, name the IRB that will review this research. If ORSP has not already agreed to rely on this IRB (via phone or email communication), you are strongly advised to contact IRBReliance@VCU.edu before proceeding with this submission:**3. * Has this study already been approved by the VCU IRB, and is now transitioning to review at another IRB (Please note, study MUST be in an approved state before it can transition to review at another IRB):**

1/7/2019

Print: HM20009089 - Maladaptive adipose tissue activity in cancer

- ☐ Yes
☒ No

ID: HM20009089

View: SF - Review Setup

Review Setup

1. * Does this study involve greater than minimal risk:

- ☐ Yes
☒ No

2. * Review Type Requested: (subject to IRB approval)

- ☐ Full Board
☒ Expedited
☐ Exempt

3. * Has this protocol received a Massey protocol review:

- ☒ Yes
☐ No

4. * Has this human subjects protocol (not the grant application) been reviewed by the funder:

- ☒ Yes
☐ No

The IRB has determined that the selected Exempt and/or Expedited categories apply to this study.

The below information is read-only to Investigators, and the categories are set by the IRB during review. All categories will appear blank until the IRB has made a determination. If a category is not checked, it does not apply to this study.

For Expedited Studies:

Category Collection of 2 Blood	Involves only the collection of blood samples by finger stick, heel stick, ear stick, or venipuncture from individuals where the amount of blood does not exceed allowable amounts (see help).
Category Specimen 3 Collection	Involves prospective collection of biological specimens for research purposes by noninvasive means.
Category Noninvasive 4 Procedures	Involves the collection of data through noninvasive procedures (not involving general anesthesia or sedation) routinely employed in clinical practice, excluding x-rays or microwaves.
Category Nonresearch 5 Data Collection	Involves materials (data, documents, records, or specimens) that have been collected or will be collected solely for nonresearch purposes including medical treatment or diagnosis.
Category Research 6 Data Collection	Involves the collection of data from voice, video, digital, or image recordings made for research purposes.
Category Behavioral 7	Is research that will be performed on individual or group characteristics or behavior OR will employ a survey, interview, oral history, focus group, program evaluation, human factors evaluation, or quality assurance methodologies.

ID: HM20009089

View: SF - Research Description

<https://irb.research.vcu.edu/irb/ResourceAdministration/Project/PrintSmartForms?Project=com.webbridge.entity.Entity%5B0ID%5BEAE13E7697DB9F...> 6/38

Research Description

1. * Describe the study hypothesis and/or research questions. Use lay language whenever possible.

The team's overall research question asks if patients with cancer and brown adipose tissue (fat) on positron emission tomography (PET) scans exhibit higher resting energy expenditure than patients with cancer without brown adipose tissue.

In Aim 1, we ask if we can identify living patients within the VCU Health System that are at least 40 years of age, have a diagnosis of cancer, and have had a PET scan demonstrating the presence or lack of brown adipose tissue.

In Aim 2, we ask if blocking the activation of BAT in patients with cancer, simply by increasing the temperature of the room they are resting in, will result in a decrease in energy expenditure. We hypothesize that blocking the activation of BAT in patients with cancer will result in a decrease in energy expenditure. This could potentially halt, or even reverse, the weight loss seen in cancer-associated cachexia, ultimately improving patients' quality of life and increase their ability to receive appropriate anti-tumor treatments.

2. * Describe the study's specific aims or goals. Use lay language whenever possible.

Aim 1: To identify patients with cancer and positive and negative brown adipose tissue on PET imaging and obtain pertinent preliminary information including MRN, zip code, age, gender, ethnicity, BMI, weight, primary cancer, diabetes status, fasting glucose data, thyroid function testing results, medication list, PET scan image and year, and brown adipose tissue status.

Aim 2: To characterize and measure the difference in energy metabolism profiles, quality of life, and biochemical profiles of patients with cancer with and without evidence of brown adipose tissue activation both at room temperature and following exposure to warm temperature.

3. * Describe the study's background and significance, including citations, or upload a citation list in document upload. Use lay language whenever possible.

Cancer associated cachexia is a condition associated with loss of muscle with or without fat loss that is associated with poor outcomes and decreased quality of life in patients with various forms of cancer (1-5). While there are many contributing factors to CAC, recent studies suggest that activation of brown adipose tissue and 'browning' of white adipose tissue may lead to increased energy expenditure and contribute to cancer associated cachexia (6-8).

See document titled 'References for Research Description' for citations.

Please see the Research protocol for more detailed background.

4. * Describe the proposed research using language understandable to those IRB committee members whose expertise is not scientific. The description must include:

- A statement explaining the study design
- A detailed description of all the procedures that will be followed to carry out the study, preferably in sequential order

If certain procedures would take place in the same manner regardless of the research (i.e. standard medical or psychological tests and procedures, routine educational practices, quality improvement initiatives, etc.), the response must clearly distinguish those procedures from procedures that are performed exclusively for research purposes or that involve alterations of routine procedures for research purposes.

If specific sections or pages of a separate protocol document are referenced that contain this detailed description, the response must include:

- a lay language overview of all research procedures
- a description of whether there are any local changes to the protocol's procedures, and if so, what those changes are (i.e. different, omitted, or additional procedures)
- any necessary clarifications of the protocol's content (i.e. what local standard of care or routine practice is)

- A description of all research measures/tests/interventions that will be used (if applicable)
- A detailed description or list of all secondary data elements and/or secondary specimens that will be obtained and how they will be used (if applicable)

See the help text for additional guidance.

Aim 1: Chart Review. This chart review requires no intervention or direct interactions with research participants, but does include accessing and recording identifiable data. Investigators will query medical records using Cerner and Montage programs for patients who are at least 40 years of age and who have cancer and have had a PET scan. The PET report will be studied to assess brown adipose tissue presence. Information to be recorded includes: MRN, zip code, age, gender, ethnicity, BMI, weight, primary cancer, diabetes status, fasting glucose data, thyroid function testing results, medication list, brown adipose tissue status (either positive or negative depending on PET image report), and PET scan image and year.

Aim 2: Energy Metabolism Profiles. We will recruit patients with cancer and brown adipose tissue activation to compare their energy expenditure with the energy expenditure of age-, sex-, BMI-, and cancer type-matched patients with no brown adipose tissue activation at room temperature. We will then assess if exposure to warm temperature, a known intervention to quench brown fat activation, is sufficient to decrease the energy expenditure in cancer patients with evidence of BAT activation. The primary endpoint in this Aim will be Energy Expenditure, measured using whole-room indirect calorimeters. The indirect calorimeter technique allows for non-invasive real-time recording of energy expenditure and respiratory quotient (a proxy for substrate utilization) by measuring oxygen consumption and carbon dioxide production. Other data that will be collected for secondary endpoints include: a quality of life questionnaire, thermal

comfort questionnaires, infrared photography, blood collection, and urine collection.

There will be three visits in total for participants that complete this study. Visit 1 will be a Screening visit, where informed consent will be obtained, inclusion/exclusion criteria will be confirmed, medical history will be obtained, physical examination will be performed, weight and vital signs will be recorded, bio-impedance analysis will be conducted, and a quality of life questionnaire will be filled out by the participant. At both visits 2 and 3, participants will have their resting energy expenditure measured by resting in the indirect calorimeters for 4 hours each time. Infrared photography from the mandible to the chest above the nipples will be taken before and after each session in the calorimeters. Telemetry physiology sensors to measure electrical activity of muscles, movements, heart rate and electrical activity, and skin temperature will be placed on the skin at the beginning of each visit and will be removed at the end of the session. Urine and blood will be collected before and after each session in the calorimeter. Thermal comfort questionnaires will be completed before and after each session in the calorimeter.

For more information, please see attached documentation.

5. Upload any supporting tables or documents (e.g. protocol documents, figures/tables, data collection forms, study communications/reminders):

ID: HM20009089

View: SF - Study Activities

Study Activities

1. * Select which study type best describes the majority of the study. Your response will help determine which IRB panel should review this.

- ☐ Bio-Medical
- ☐ Qualitative - Social/Behavioral/Education (SBE)
- ☐ Quantitative - SBE
- ☐ Mixed Method - SBE
- ☒ **Mixed Method - Biomedical**

2. * This study will involve (check all that apply):

- ☒ **procedures such as surveys, interviews, field studies, focus groups, educational tests, deception, psycho-physiological testing, any other similar data collection**
- ☒ **secondary data analysis: procedures such as analysis of information collected for non-research purposes (includes both retrospective and prospectively collected information), or analysis of data previously collected for a prior research study**
- ☒ **drugs, devices, experimental interventions, biohazards, radiation, other medical or surgical procedures**

ID: HM20009089

View: SF - Bio-Med Project Details

Bio-Med Project Details

1. * Select all that apply to this study:

- ☐ Drugs, Biologics, Supplements, and/or Other Compounds
- ☐ Placebo
- ☐ Washout Period
- ☐ Device Evaluation
- ☐ Bio-Hazards, Other Toxins, Recombinant DNA/Gene Transfer
- ☐ Radiation Exposure and/or Scans involving radiation (eg: PET, MRA, CT, DXA, nuclear medicine, etc)
- ☐ Stem Cells
- ☐ Expanded Access - Treatment Use of an Investigational Product
- ☒ **Other Medical or Surgical Procedures (eg: physical exam, sample collection, clinical procedures, scans, etc)**
- ☒ **Protected Health Information (PHI)**
- ☐ None of the Above

Social/Behavioral Project Details

1. * Select all that apply to this study:

<input checked="" type="checkbox"/>	Analysis of Information Originally Collected for Non-Research Purposes
<input type="checkbox"/>	Analysis of Data Originally Collected for a Previous Research Study
<input type="checkbox"/>	Behavioral Intervention or Experimentation
<input type="checkbox"/>	Observations
<input type="checkbox"/>	Educational Settings/Assessments/Procedures
<input type="checkbox"/>	Population Based Field Study
<input type="checkbox"/>	Psychophysiological Testing
<input type="checkbox"/>	Deception
<input type="checkbox"/>	Oral History
<input type="checkbox"/>	Interview/Focus Groups
<input checked="" type="checkbox"/>	Surveys/Questionnaires/Psychometric Testing
<input type="checkbox"/>	None of the Above

2. * Will any portion of the research be potentially upsetting to the participants:

- ☐ Yes
☒ No

3. If Yes, describe the nature of the questions and how you will manage the situation should participants become upset:

4. Upload ALL Instruments/guides that will be used, including scripts/questions to guide Interviews, surveys, questionnaires, observational guides, etc.:

Surveys/Questionnaires

1. * Does your study involve a satisfaction survey administered to VCUHS patients (*See Help Text):

- ☐ Yes
☐ No
☒ Not Applicable

Data Collection Details

1. *

Select all involved in the study:

<input checked="" type="checkbox"/>	Specimen/Biologic Sample Collection
<input checked="" type="checkbox"/>	Protected Health Information (PHI)
<input checked="" type="checkbox"/>	Secondary Data or Specimens Not From a Registry or Repository
<input type="checkbox"/>	Audio/Video

☐ Use of Internet for Data Collection

☒ Registries/Repositories (Includes Accessing, Contributing or Creating)

☐ None of the Above

2. * Select all Identifiers that will be collected as part of this study (including for recruitment, data gathering, data analysis, etc.), even if the data will eventually be anonymized:

☒ Names

☒ Geographic Locators Below State Level

☒ Social Security Numbers

☐ Dates (year alone is not an identifier)

☒ Ages >89

☒ Phone Numbers

☐ Facsimile Numbers

☒ E-mail Addresses

☒ Medical Record Numbers

☐ Device Identifiers

☐ Biometric Identifiers

☐ Web URLs

☐ IP Addresses

☐ Account Numbers

☐ Health Plan Numbers

☐ Full Face Photos or Comparable Images

☐ License/Certification Numbers

☐ Vehicle ID Numbers

☐ Other Unique Identifier

☐ No Identifiers

☐ Employee V#

3. If "Other Unique Identifier" was selected above, describe the identifiers:

.

4. * Will participants be able to withdraw their data (paper, electronic, or specimens) from the study if they no longer wish to participate:

☐ Yes

☒ No

5. If yes above, describe how participants will be able to withdraw their data:

Aim 1: Participants will not be able to withdraw their data from the study if they no longer wish to participate, as it is just a chart review and we have a waiver for consent for this portion of the study.

Aim 2: Patients will be allowed to withdraw from the study at any time, as outlined in the PRMC protocol attached in Section 5.2. If a participant withdraws, they can choose to withdraw their samples from any future studies for data collection, but data that has already been collected before withdrawal will not be deleted.

ID: HM20009089

View: SF - Sample Collection

Sample Collection

1. * Select all of the types of samples that will be collected as part of this study:

☐ Amniotic Fluid

☒ Blood

☐ Buccal Smears

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<input type="checkbox"/>	Saliva
<input type="checkbox"/>	Tissue
<input checked="" type="checkbox"/>	Urine
<input type="checkbox"/>	Other
<input type="checkbox"/>	None of the Above

2. If Other, please describe the type of sample being collected:

3. * Describe how the samples will be collected and the collection schedule. For each type of sample, include information about

- The procedures that will be followed to collect the sample
- The role(s) of the individuals who will collect the sample
- The volume/size range of the sample
- The timing and frequency of sample collection

Approximately 12mL of blood will be collected in tubes with no additives except serum separator before and after each session. No more than 50ml of blood will be collected over the course of the two sessions. Serum will be separated by centrifugation, aliquoted in 1 mL tubes, and stored in -80°C freezers in Dr. Cell's laboratory in Sanger Hall. Samples will be analyzed for glucose, lipids, free fatty acids, exosomes, and other components for hormonal and metabolic factors related to metabolism, cancer, and cancer-associated cachexia. Samples will be stored as described above for future analyses in the registry we are creating, if that participant consents, for research for medical problems.

At each energy expenditure recording session, urine will be collected before and after the recording to allow for a timed collection. Urine samples will be analyzed for creatinine, cortisol, electrolytes, catecholamines, and metanephrines. Urine aliquots will be stored in -80°C freezers in Dr. Cell's laboratory in Sanger Hall for future analyses in the registry we are creating, if that participant consents, for research for medical problems.

4. * Will Genetic Testing be conducted on any of the samples:

- ☒ Yes
☐ No

5. * Will any of the samples be used for a pregnancy test:

- ☐ Yes
☒ No

6. If yes, describe how positive pregnancy results will be communicated to the participant, particularly if minors are involved:

7. * Will any of the samples be used to screen or document alcohol or illicit drug use:

- ☐ Yes
☒ No

8. * I am aware that I may need to establish a research account with VCUHS Department of Pathology for specimen processing:

- ☒ Yes
☐ No

ID: HM20009089

View: SF - Blood Details

Blood Details

1. * Select all of the methods of sample collection that will be utilized in this study:

<input type="checkbox"/>	Individual Needle Stick(s)
<input checked="" type="checkbox"/>	Indwelling Catheter Placed Solely for This Study
<input checked="" type="checkbox"/>	Indwelling Catheter Placed for Other Reason(s)
<input type="checkbox"/>	Blood Collected at the Same Time as Non-Research Blood Collection(s)
<input type="checkbox"/>	Unused Blood Originally Drawn for Clinical Purposes
<input type="checkbox"/>	Other

ID: HM20009089

View: SF - Urine Details

Urine Details

1. * In order to collect urine, will an indwelling catheter be placed solely for the research study:

☐ Yes
☒ No

ID: HM20009089

View: SF - DNA/Genetic Testing Details

DNA/Genetic Testing Details

1. * What are the intended research areas that this DNA will be collected for:

DNA will be collected for research in metabolism, cancer, and/or cancer-associated cachexia. We will also keep DNA in our registry for future use, if the participant consents, for research in any medical problems.

2. * Is the genetic component of the study optional (i.e., participants can take part in the research without participating in the genetic testing):

☒ Yes
☐ No

3. * Will any tests be conducted that could create clinically relevant findings (identify conditions that could be treated or where the participant could benefit from genetic counseling)?

☐ Yes
☒ No

4. * Will genetic testing results, including paternity, be provided to the participants:

☐ Yes
☒ No

5. If yes, address the following:

- whether tests will be run in a CLIA-certified laboratory
- the procedure for providing results to participants
- whether participants will have the option to decline results
- how participants might access genetic counseling for assistance in understanding the implications of genetic testing results
- any costs to participants (e.g., for genetic counseling):

6. If no, results will not be provided, explain why not:

We are not screening for any genetic diseases, and any genetic testing we do will be for research purposes, so we explain this in our consent form.

7. * Will participants have the option to request that samples and/or test results be withdrawn in order to prevent further analysis, reporting, and /or testing for this study:

☒ Yes
☐ No

8. If Yes, describe the following:

- how participants will be informed of how to withdraw samples or data
- how participants will be informed that their request has been granted
- whether participants will be able to continue with non-genetic components of the study:

In our consent form, we make it clear that patients can participate in the non-genetic components of the study regardless of whether they consent to the genetic testing. We also make it clear that they can withdraw their consent at any time - in this case, if they withdraw consent, any prior data collected will still be kept, but no future analyses will be performed.

9. * Will the DNA be stored for use in future research:

☒ Yes
☐ No

ID: HM20009089

View: SF - HIPAA

HIPAA

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1. * Describe the protected health information that will be obtained or used in this research:

This chart review will require access to full medical records.

2. * Describe the source(s) of the protected health information:

Aim 1: Cerner and Montage programs at VCU will be the source for the PHI collected in this chart review.

Aim 2: additional PHI will be collected from Cerner, participant self-reporting, and study procedures.

3. * Explain how the PHI collected or used in this research is the minimum necessary to accomplish this research:

Aim 1: The information we will collect includes: MRN, zip code, age, gender, ethnicity, BMI, weight, primary cancer, diabetes status, fasting glucose data, thyroid function testing results, medication list, PET scan image and year, and brown adipose tissue status. These are the minimum items necessary to determine the number of patients at VCU who would be of interest for a subsequent clinical study, as it is crucial to match participants using these factors (age, gender, BMI, brown adipose tissue activity/presence, and type of cancer can all contribute to cancer associated cachexia in different ways, so we need to have this information. PET scan images are necessary because it is important to visually discern differences in brown adipose activity in individuals who are positive and negative for brown adipose tissue on their PET scan. Ethnicity is important to document, as there may be differences in energy expenditure, cancer outcomes, etc between different ethnic groups. We also need weight because BMI is not always the best assessment of an individual's metabolic state. Medications are important for us to document, as certain medications such as beta blockers are known to be involved in pathways associated with thermogenic activity.

Aim 2: We are collecting the information as in Aim 1 as well as contact information such as phone number and email address, which are necessary for communicating with participants to coordinate their study visits.

4. * Select all pathways this research will employ to use or access PHI:

- ☐ De-Identified Data (none of the 18 identifiers are recorded or associated with the research data)
- ☐ Limited Data Set
- ☒ **Waiver of Authorization**
- ☐ Partial Waiver of Authorization
- ☒ **Signed Authorization Combined with Consent Form**
- ☐ Signed Authorization as Stand-Alone Form

ID: HM20009089

View: SF - Waiver of Authorization

Waiver of Authorization

1. * Describe how the use of PHI in this study poses no greater than minimal risk to subject's privacy:

PHI obtained poses no greater than minimal risk to subject's privacy as there will be no participant interactions or interventions that would put their privacy at risk.

2. * When will identifiers be destroyed:

The identifiers collected in this protocol will be destroyed after the completion of the analysis and publication of the data.

3. * Other than the PI and research personnel identified in this application, who else will have access to the Protected Health Information?

Only the PI and Research Personnel in this application will have access to the PHI collected.

4. * Explain why the research cannot practicably be conducted without the waiver of authorization:

Obtaining authorization for each chart review is not feasible in this case, as we aim to go through a large number of charts in order to narrow our focus for a subsequent study. Contact for each chart review would exceed the time available by study staff time, as we expect to review charts for an estimated 1500 patients (see Study Population section). This chart review only involves reviewing medical record information and will not involve direct engagement of or interaction with participants; in the subsequent study, we will not need a waiver of authorization, as this current chart review will narrow our potential sample size and will be within the scope of our resources.

5. * Explain why the research cannot practicably be conducted without access to and use of the Protected Health Information (PHI).

All of the proposed PHI for data collection (Geographical location, age, gender, ethnicity, BMI, weight, primary cancer diagnosis, diabetes status, fasting glucose data, thyroid function testing results, medication list, PET scan brown adipose tissue status, PET scan image and year, and MRN) are the necessary to carry out the goals of this proposal. Since age, gender, ethnicity, weight, BMI, brown adipose tissue status, thyroid function, medications, and primary cancer diagnosis may contribute to cancer associated cachexia, this PHI must be used. PET scan image must be access and used because it is critical to have visual representations of differences in brown adipose tissue in this patient population. MRN is necessary to ensure that we are collecting the appropriate data for each individual. Contact information is necessary to communicate with the participants and coordinate study visits.

6. * In applying for a waiver of authorization, the PI agrees to the following:

- the identifiers used for this research study will not be used for any other purpose or disclosed to any other person or entity (aside from members of the research team identified in this application), except as required by law.

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- if at any time the PI wants to reuse this information for other purposes or disclose the information to other individuals, the PI will seek approval from the IRB/Privacy Board.
- that the PI will comply with VCU HIPAA policies and procedures and to the use and disclosure restrictions described above.
- the PI assumes responsibility for all uses and disclosures of the PHI by members of the study team.

☒ Yes
☐ No

ID: HM20009089

View: SF - Existing Data/Specimen Details

Existing Data/Specimen Details

1. * Describe the source and nature of the data/specimens being obtained:

Data will be coming from a chart review. The chart review requires no intervention or direct interactions with research participants, but does include accessing and recording identifiable data. We will query medical records using Cerner and Montage programs for patients who are at least 40 years of age and who have cancer and have had a PET scan. The PET report will be studied to assess brown adipose tissue presence. Information to be recorded includes: MRN, zip code, age, gender, ethnicity, BMI, weight, primary cancer, diabetes status, fasting glucose data, thyroid function testing results, medication list, PET scan image and year, and brown adipose tissue status (either positive or negative depending on PET image report).

2. * Describe how you have access to the data/specimens:

We will be conducting the chart review and collecting the data.

3. *

Describe any identifiers or coded information that will be obtained that can be linked directly or indirectly to the identity of participants:

We will be collecting PHI but will be coding information to minimize risk to the patients. Please see Data Confidentiality section for this information

4. * Did individuals provide consent for research when the data / samples were originally collected?

☐ Yes
☒ No

5. If yes, did the consent allow for sharing of the data:

☐ Yes
☐ No

ID: HM20009089

View: SF - Registry/Repository Details

Registry/Repository Details

1. * Select all that apply:

- ☐ Contributing to an Existing Registry or Repository
- ☒ **Creating a New Registry or Repository**
- ☐ Submitting Data to a NIH genomic data repository
- ☐ Retaining de-identified data or biospecimens for future research.
- ☐ Accessing a Registry or Repository (Usage Protocol)

2. Provide the general purpose and target area(s) of study for the Registry/Repository:

The general purpose of this registry is to have serum, urine, and DNA stored for study of diseases related to metabolism and cancer.

ID: HM20009089

View: SF - New Registry/Repository Details

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New Registry/Repository Details

1. Provide name of registry or repository, if applicable:

Cancer and Metabolism Registry

Expected start date: 07/2018

On-going. Dr. Celi will be the leader of this registry and if leadership must change, a new leader will be added to the registry protocol before switching over.

2. * Site having responsibility for the management of this registry/repository:

☒ VCU

☐ Non-VCU

3. If VCU is not responsible for the management of this registry / repository describe the organization and/or individual who is responsible:

4. * Describe in detail how access and control of the registry/repository will be managed:

Only individuals listed on this IRB protocol will have full access to registry samples and data. Password protected files will be used for data and only IRB-approved personnel will have access.

Any investigators who wish receive data or samples from this registry must have their own IRB approval indicating that they will have de-identified data provided by us.

5. * Describe any identifiers (including linkable codes) that will accompany data or samples to the registry/repository:

We will have two files: one file, the "key", with MRN, randomly assigned number, and participant contact information; and the other file, the "data file", with the randomly assigned number and accompanying data.

The "Key" file will be password protected and will only be accessible to the personnel in this IRB protocol.

6. * Describe how other researchers will be able to request access to the data/samples contained in the registry/repository for future research.

A request in writing must be submitted to Dr. Celi with documentation that includes: the researcher's study objective/aims, the protocols which they wish to perform, and regulatory approval if applicable.

7. * Describe how coded or identified data will be released to others:

As stated above, each individual participant will be assigned a unique study number. and the data and samples will be deidentified. The file containing the key will be maintained separated in the PI (Dr. Celi) office. Other researchers will only be given anonymous data and will never have access to the key.

8. * What safeguards will be in place to prevent accidental or inappropriate release of information:

All individuals who will have access to information must have appropriate training in responsible research. This includes HIPPA training, CITI training (or equivalent), etc.

Other researchers interested in studying the registry/repository will contact the PI (Dr. Celi). Each request will be filed in a dedicated folder. A log containing the following information will be created:

1) Name, contact and organization of the requester.

2) Purpose of the study.

3) List of the data shared.

4) Type and amount of samples shared.

Data and samples will be anonymized.

The registry/repository will be utilized exclusively for the purpose of studying metabolism in patients affected by cancer.

The PI (Dr. Celi) will be the solely responsible for the decision of sharing data and samples with other researchers.

9. * Describe the conditions under which information or specimens contained in the registry/repository may be released to other researchers:

The PI (Dr. Celi) will be responsible for sharing data and samples with other investigators following a formal request stating the objective of the study and type of research/analysis. Since data and samples will be deidentified, the risk of dissemination of PHI is negligible. Additionally, other researchers must state in their protocols that they will not attempt to re-identify participants, that they will not share the data/specimens with any other researchers, and that they will return any left over sample at the end of their study. Dr. Celi and the other researchers will discuss coauthorship or acknowledgement in any future publications, depending on Dr. Celi's involvement with the proposed projects.

10. * How long will the registry/repository be maintained?

We foresee to expand this project in a larger research. To this end, we expect to maintain the data and samples for a period of up to ten years. Should the research be abandoned sooner we will close the registry and dispose the samples in the repository.

11. * If participants will be able to access their data and/or samples from the registry/repository for personal use, explain how this will occur.

Study participants will not be able to access the data and samples for personal use.

12. * Explain how participants are allowed to request the data/samples be destroyed/removed from the registry/repository or why it is not allowed:

Study participants can revoke the participation in Aim 2 of the study by sending a written request to the PI (Dr. Celi). Their data will be

removed from the registry and remaining samples will be disposed.

Study participants will be made aware that data already published prior their request to be removed from the registry/repository cannot be amended.

13. If the participant will be stipulating future use for this data/specimens, address 1) what are the stipulations and 2) describe the registry/repository mechanism to capture and utilize these stipulations.

Our consent form has the following set of Yes/No questions for participants to fill out:

1. My blood samples may be used for genetic testing. I am aware that the results of the genetic testing will not be provided to me.
2. My blood samples may be stored and used for future research about cancer and other metabolic diseases.
3. My blood samples may be stored and used for future research about other medical problems.
4. My urine samples may be stored and used for future research about cancer and other metabolic diseases.
5. My urine samples may be stored and used for future research about other medical problems.

In our data collection sheets, we will have columns corresponding all 5 of these questions, and will fill each with the selected 'Yes' or 'No' choice selected by each patient for each question. For those who consent to at least one of these, their information will also be included in the registry file, which will again have the columns and corresponding yes/no answers so that it is clear what the participant did and did not consent to regarding future use of their specimens. Please refer to Question 3 of Data Confidentiality and Storage for information on the coding system.

14. If there is not a mechanism to capture the participants data use stipulations, explain why this is not necessary:

15. * If the Informed consent will offer an option for participants to be contacted prior to any future use, describe the procedure(s) for contacting participants regarding future use or studies.

N/A.

ID: HM20009089

View: SF - Data Confidentiality and Storage

Data Confidentiality and Storage

Confidentiality refers to the way private, identifiable information about a participant or defined community is maintained and shared.

1. Using the VCU Data Classification Tool, please determine the appropriate data classification category for the data that will be collected or used in this research. Note: If the data falls into Category 1, a data security management plan is required by University Information Security Office.

See help text for information on accessing the VCU Data Classification Tool, and for information on creating a data security management plan:

- ☒ **Category 1: all data that require breach notifications in the event of improper release, including all non-publicly available personally identifiable information covered by HIPAA and Commonwealth of Virginia regulations.**
- ☐ **Category 2: all proprietary data that if improperly released has the potential to cause harm to the institution, its mission or its reputation that do not require breach notifications.**
- ☐ **Category 3: all non-proprietary data that is considered publicly available for unrestricted use and disclosure. Such information is available to all members of the University community and to all individuals and entities external to the University.**

2. * I confirm use of the VCU Data Classification Tool in determining the data classification category selected in Question 1:

- ☒ **Yes**
- ☐ **No**

3. * Specify where this study's paper and electronic research data and/or physical specimens will be stored and how they will be secured from improper use and disclosure:

Several precautions will be used to maintain data confidentiality. First, data will be kept in electronic format to eliminate paper storage and associated risks. Second, access to data will be limited exclusively to the individuals listed on the IRB protocol, as all are essential to the study. Lastly, we will use both REDcap and password-protected files stored on the VCU SOM server, both of which are approved for use with HIPAA data, to collect, record, analyze, and store our data.

Physical specimens will be stored at -80C in freezers in the key-lock-protected lab of the PI in Sanger Hall. Specimen labels will have code numbers and no PHI to protect from improper disclosure.

Consent forms and other paper documents will be stored in a locked cabinet in the PI's key-lock-protected office.

4. * Who will have access to study data:

The PI, co-investigators, and student will have access to this data.

5. * If the study will code (i.e. de-identify) the research data by replacing subjects' names with assigned subject IDs, explain the following aspects of the coding process:

1. The process for how subject IDs will be generated/assigned (e.g. random, sequential)
2. Whether there will be a key that links the subject ID with direct identifiers.

If a key will be created, describe

3. The place where the key will be stored
4. The role(s) of all individuals who will have access to the key
5. When the key will be destroyed

See the help text for additional guidance.

We will have separate files for Aim 1 and Aim 2. We will also have another file just for registry participants.

Aim 1:

Codes will be assigned as to each MRN as it is reviewed. Codes will be in the form of a numerical value ranging from 1 to 5000 (5000 was chosen because it is much larger than our anticipated sample size (1500) in Aim 1 in this study). A random number generator will be used to generate a column of random numbers without duplicates in this range. This column of random numbers will be copied into an excel file and will be assigned to MRNs as they are reviewed. MRNs and their associated codes will be saved in a password-protected excel file saved on REDcap and will be the key document. A separate password-protected excel file in REDcap will include the other information we will collect for each individual (zip code, age, gender, BMI, primary cancer diagnosis, diabetes status, fasting glucose data, thyroid function testing results, PET scan image (with no identifiers on the image), brown adipose tissue status) along with the code associated with their MRN. Access to the key will be limited to IRB personnel. The key will be destroyed 5 years after publication of study results.

Aim 2:

Codes will be assigned to each MRN as participants consent to the study. Although they will have had a code in Aim 1, we will be creating a new code for Aim 2. Codes will be in the form of a numerical value ranging from 1 to 100 (100 chose because it is larger than our targeted enrollment of 36 participants) and numbers will be randomly generated. As in Aim 1, the MRNs and their associated codes will be saved in a password-protected excel file saved on REDcap and will be the key document for Aim 2. A separate password-protected excel file in REDcap will include the other information we will collect for each individual (zip code, age, gender, BMI, primary cancer diagnosis, PET scan image (with no identifiers on the image), brown adipose tissue status) the code associated with their MRN, and any information collected during the study protocol. Additionally, there will be columns indicating whether or not each participant consented to genetic and registry testing. Access to the key will be limited to IRB personnel. The key will be destroyed 5 years after publication of study results.

For those who consent to the registry, a separate file will be created using the same key code from Aim 2's file, but only containing the information from participants who consented for the registry. There will be a column to indicate whether or not each participant consented to genetic testing. Access to the key will be limited to IRB personnel. The key will be destroyed 5 years after the closure of the registry.

6. * Will the sponsor or investigator obtain a certificate of confidentiality for this study:

- ☐ No - Will not obtain CoC for this study
- ☐ Yes - CoC has been obtained or issued automatically
- ☐ Yes - CoC request is Pending
- ☒ Yes - Plan to submit request for CoC

7. If the Certificate of Confidentiality has been obtained by the PI, upload it here:

Document Name	Document	Version	Date Modified	Uploaded By	Type	Approved
View CV_Stamatouli	StamatouliCVsigned.pdf	0.01	11/20/2018 8:33 AM	Janina Pearce	CV/Biosketch	Not Applicable
View Consent_CoC	2018.07 Consent.pdf	0.12	8/8/2018 10:35 AM	Janina Pearce	Consent/Assent/Information Sheet	Yes
View Protocol_PRMC	2018.07 PRMC.docx	0.06	7/24/2018 12:12 PM	Janina Pearce	Research Protocol	Yes
View Email script	2018.04 Email contact.docx	0.01	4/30/2018 8:15 AM	Janina Pearce	Recruitment/Advertising	Yes
View Phone script	2018.04 Phone contact.docx	0.01	4/30/2018 8:15 AM	Janina Pearce	Recruitment/Advertising	Yes
View TelegRAM	2018.04 TelegRAM.docx	0.01	4/30/2018 8:15 AM	Janina Pearce	Recruitment/Advertising	Yes
View StudyFlyerRecruitment	2018.04 Recruitment Flyer.docx	0.02	4/30/2018 8:12 AM	Janina Pearce	Recruitment/Advertising	Yes
View EnergyExpenditureLetter_Example	2018.04 ParticipantResults.docx	0.01	4/25/2018 7:28 AM	Janina Pearce	Other	Yes
View MasseyPilotFunding	19. Funding Notice Celi_Chen_Del Fabbro.pdf	0.01	2/23/2018 11:53 AM	Janina Pearce	Funding Proposal	Not Applicable
View Questionnaire_ThermalComfort	Thermal Comfort Questionnaire.pdf	0.01	2/23/2018 8:45 AM	Janina Pearce	Research Measure	Yes

1/7/2019

Print: HM20009089 - Maladaptive adipose tissue activity in cancer

	Document Name	Document	Version	Date Modified	Uploaded By	Type	Approved
View	Questionnaire_QualityOfLife	QoL FAACT Questionnaire.pdf	0.01	2/23/2018 8:45 AM	Janina Pearce	Research Measure	Yes
View	Chen CV	SChenCV.pdf	0.01	2/20/2018 10:27 AM	Janina Pearce	CV/Biosketch	Not Applicable
View	MCC_PRMC_ProspectiveStudy Letter	2018-01-08_MCC-17-13470_PRMC Approval Letter.pdf	0.01	2/20/2018 10:26 AM	Janina Pearce	Ancillary Committee Approval	Not Applicable
View	MCC_PRMC_ChartReview Letter	2017-01-17_MCC-17-13218_PRMC Review.pdf	0.01	1/25/2017 8:51 PM	Francesco Celi	Ancillary Committee Approval	Not Applicable
View	References for Research Description	References for Research Description.docx	0.01	11/11/2016 9:13 AM	Janina Pearce	Research Protocol	Not Applicable
View	Del Fabbro CV	Del Fabbro CV 2016 oct.pdf	0.01	11/2/2016 9:36 AM	Janina Pearce	CV/Biosketch	Not Applicable
View	Celi CV	FS Celi CV August_2016_VCU.doc	0.01	11/2/2016 9:36 AM	Janina Pearce	CV/Biosketch	Yes
View	Vaitkus Biosketch	Vaitkus_Biosketch2.pdf	0.01	10/31/2016 12:36 PM	Janina Pearce	CV/Biosketch	Yes

8. * What will happen to the research records when the research has been completed:

- ☒ Stored indefinitely with identifiers removed
- ☐ Stored indefinitely with identifiers attached
- ☐ Destroyed at the end of study once the minimum time required for data retention has been met per VCU Data Retention Policy and/or sponsor retention requirements
- ☐ Destroyed when notified by sponsor but not less than the minimum time required for data retention per VCU Data Retention Policy
- ☐ Other

9. If Other, explain:

10. If "stored indefinitely with identifiers attached", explain why identifiers are necessary:

ID: HM20009089

View: SF - Types of Sites

Types of Sites

1. * Select which of the following accurately describes this study:

- ☒ Not Multi-site Study
- ☐ Multi-site Study [multiple sites implementing the same protocol] - VCU Lead
- ☐ Multi-site Study [multiple sites implementing the same protocol] - Non-VCU Lead

2. * Select all sites where study interventions or interactions will occur and/or identifiable data will be held:

- ☒ VCU Site
- ☐ Non-VCU Site (VCU Investigators are conducting/overseeing the conduct of the study)
- ☐ Non-VCU Site under the oversight of a Non-VCU PI

3. * Is there a community partner in this research study:

- ☐ Yes
- ☒ No

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View: SF - VCU Site Details

VCU Site Details

1. * Select all VCU sites that will be utilized in this study:

<input type="checkbox"/>	Children's Hospital of Richmond at VCU
<input checked="" type="checkbox"/>	Clinical Research Services Unit (CRSU)
<input checked="" type="checkbox"/>	Massey Cancer Center
<input type="checkbox"/>	VCU Health Community Memorial Hospital
<input checked="" type="checkbox"/>	VCU Medical Center downtown
<input type="checkbox"/>	VCU Monroe Park Campus
<input type="checkbox"/>	VCU Qatar
<input type="checkbox"/>	Other VCU Site

2. * Provide details regarding each VCU Site including:

- what clinics / facilities will be used

- resources that are available for the conduct of this study:

Aim 1: Since this Aim is a Chart Review, we will be accessing medical records in the system using Cerner and Montage.

Aim 2: Study sessions will take place in the CRSU, which has dedicated resources that will allow us to carry out our study.

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View: SF - VCU Health System

VCU Health System

1. * The PI has reviewed and agrees to comply with the Conduct of Clinical Research in VCU Health System Patient Care Areas policy:

- ☒ Yes
☐ No

2. * Explain how you will notify and obtain support from patient care providers in the units where the study will be conducted:

Aim 1: The chart review will be done electronically/without participant interaction, so there is no need for support from care providers.

Aim 2: Dr. Del Fabbro will be our main point of contact for patients in this study, and he will be accessible for notification and support should the need arise for these participants with cancer.

ID: HM20009089

View: SF - Study Funding

Study Funding

1. * Have you applied for funding:

- ☒ Yes
☐ No

2. If so, is this study already funded:

- ☒ Yes
☐ No

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View: SF - Funding Details

Funding Details

1. * Select all funding sources for this study (pending or awarded):

<https://irb.research.vcu.edu/irb/ResourceAdministration/Project/PrintSmartForms?Project=com.webbridge.entity.Entity%5B0ID%5BEAE13E7697DB9...> 19/38

<input type="checkbox"/>	Industry
<input type="checkbox"/>	Direct Federal
<input type="checkbox"/>	Non-Profit
<input type="checkbox"/>	Indirect Federal
<input type="checkbox"/>	State/Local Government
<input type="checkbox"/>	Internal Grant
<input type="checkbox"/>	Investigator/Departmental Funds
<input type="checkbox"/>	None
<input checked="" type="checkbox"/>	Other

2. Since Other was selected above, describe the source(s) of funding for this study:
Massey Pilot Funding. We are attaching the funding letter as a pdf.

3. Select all related proposals:
RAMS-SPOT ID# (FP/PT/PD#)
There are no items to display

Sponsor	PI	Title	Status	Start	End
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ID: HM20009089

View: SF - Non-Direct Federal Funding

Non-Direct Federal Funding

1. * Does the funder require congruence review of this proposal:

- ☐ Yes
☒ No

2. Documents required for upload:

- If funder requires congruence review, upload the entire grant proposal (exclusive of budget and appendices).
- If the funder requires a specific form to be completed upload this form here.

ID: HM20009089

View: SF - Study Population

Study Population

1. * Provide the total number of Individuals at VCU, and at other sites under the VCU IRB, that:

1. May participate in any study interaction or intervention (including screening, consenting, and study activities) AND/OR
2. You may obtain any data/specimens about (regardless of identifiability)

See the help text for additional guidance.

1500

2. If this is a multi-Center Project, what is the total anticipated number of subjects across all sites:

3. * Provide justification for the sample size:

Aim 1: This is chart review, so a large number of charts is expected within the system. Nuclear medicine performs approximately 300 PET scans/year for patients with cancer. Per observations from Radiology, approximately 50 of those exhibit some brown fat expression. We are interested in identifying as many patients with brown fat expression as possible, so we expect to go through a large number of charts. Please note that 1500 listed in question 1 is an estimation, and not an exact number.

Aim 2: We are expecting to enroll about 36 participants for this portion of the study. See PRMC protocol for more details.

4. * List the study inclusion criteria:

We are requesting access to all patients in Cerner and Montage to search for our particular patient cohort. Inclusion criteria for recording information will include:

<https://irb.research.vcu.edu/irb/ResourceAdministration/Project/PrintSmartForms?Project=com.webbridge.entity.Entity%5B0ID%5BEAE13E7697DB9...> 20/38

Living
 ≥ 40 years of age
 Have active cancer
 Have had a PET/CT scan within the past 36 months at time of initial contact
 Ability to understand and the willingness to sign a written informed consent document.

Date range for charts to be included: 10 years from approval of protocol

5. * List the study exclusion criteria:

Aim 1: Criteria that will exclude participants from having their information recorded will include:

Deceased
 Less than 40 years of age at the time of PET scan
 Prior cancer diagnosis that has been treated to remission (by any means, including surgery, radiation, chemotherapy)
 No PET scan or reading available

Aim 2: Exclusion criteria are:

Current use of beta-blockers
 Women who are pregnant or unsure of their pregnancy status
 Women who are breastfeeding
 Suffers from severe claustrophobia
 Diagnosed with a serious psychiatric condition which could impede the judgement of the investigators, and/or the successful conduct of the recording.
 In remission stage for cancer diagnosis

6. * Check all participant groups that will be included in this study or discernable in the research data/specimens. In particular, if you will know that a regulated vulnerable population (children, pregnant women, or prisoners) is involved in the study, be sure to check them:

- ☐ Healthy volunteers
- ☐ Children
- ☐ Emancipated minors
- ☐ Pregnant women
- ☐ Fetuses, Neonates, Post-delivery Materials, or In-Vitro Fertilization
- ☐ Prisoners
- ☐ Decisionally Impaired Adults
- ☒ When cancer is integral to the research - cancer patients, their family members, cancer healthcare providers, or cancer prevention
- ☒ VCU Health System or VCU Dental Care patients
- ☐ Non-VCU patients
- ☐ VCU / VCUHS students or trainees
- ☐ VCU / VCU Health System employees
- ☐ Individuals with limited English proficiency
- ☐ Active military personnel
- ☐ When researching in a K-12 environment - populations within school districts or other learning environments

7. * Does this study obtaining data in, or from, the European Economic Area? (see Help Text for list of countries included in the EEA)

☐ Yes ☒ No

8. Justify the inclusion and exclusion criteria if necessary. If you are either targeting, or excluding, a particular segment of the population / community, provide a description of the group/organization/community and provide a rationale:

The inclusion/exclusion criteria listed above are necessary due to the nature of our research question and to maximize patient protection. Targeting patients with current/active cancer is necessary in the context of cancer associated cachexia. PET scan imaging is standard of care in cases of cancer, so this should not exclude particular segments of the community who have cancer.

9. * Select the age range(s) of the participants who may be involved in this study:

- ☐ < 1 Year
- ☐ 1 - 6 Years
- ☐ 7 - 12 Years
- ☐ 13 - 17 Years
- ☐ 18 - 20 Years
- ☒ 21 - 65 Years
- ☒ > 65 Years

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View: SF - Cancer Related Studies

Cancer Related Studies

1. * All studies targeting cancer patients, family members of cancer patients, cancer healthcare providers, or cancer prevention where cancer is integral to the research question require review by Massey Cancer Center Protocol Review and Monitoring Committee (PRMC). Upload documentation of PRMC approval.

ID: HM20009089

View: SF - Potential Subject Identification and Recruitment

Potential Subject Identification and Recruitment

1. * Choose all recruitment methods that may be used:

<input type="checkbox"/>	E-mail Campaign
<input type="checkbox"/>	Phone Solicitation
<input checked="" type="checkbox"/>	Flyers, Letters or Newspaper/TV/Radio Ads
<input type="checkbox"/>	Website
<input checked="" type="checkbox"/>	Direct Contact
<input type="checkbox"/>	Psychology Research
<input type="checkbox"/>	Participant Pool (SONA)
<input checked="" type="checkbox"/>	VCU TelegRAM announcement
<input type="checkbox"/>	Word of Mouth
<input checked="" type="checkbox"/>	Other

2. If Other, please describe:

Aim 1: comprehensive chart review using Cerner and Montage programs to determine potential participants for a future study and document PHI (explained elsewhere in this submission).

Aim 2: Flyers, TelegRAM, and direct contact by Dr. Del Fabbro (either through his existing relationship with participants, or by referral by another health care professional).

3. * Select the methods used to obtain names and contact information for potential subjects:

<input checked="" type="checkbox"/>	Pre-Existing Relationship with Participants
<input checked="" type="checkbox"/>	Selected from Pre-Existing VCU Records
<input type="checkbox"/>	Selected from Pre-Existing Non-VCU Records
<input type="checkbox"/>	Selected from Publicly Available Records
<input checked="" type="checkbox"/>	Referred by Health Care Provider or Other Health Professional
<input type="checkbox"/>	Recruited from Database or Registry
<input type="checkbox"/>	Identified through Community Based Organization (Schools, Church Groups, etc.)
<input type="checkbox"/>	Self Referred (Flyer/Ad)
<input type="checkbox"/>	Other

4. If Other, please describe:

5. * Provide a description of:

1. How potential participants or secondary data/specimens of interest will be identified and

2. All procedures that will be followed to carry out recruitment and screening activities.

Include details (as applicable) about:

- How secondary data/specimens that meet the study's eligibility criteria will be identified (i.e. what database(s) will be queried and the search terms that will be used)
- How potential participants will be identified and their contact information obtained

- The timing and frequency of recruitment activities
- Where and how recruitment procedures will be completed
- Who will recruit or respond to potential participants
- What and how written or verbal recruitment materials and reminders (if any) will be used
- What screening activities will occur and how these procedures will be performed

See the help text for additional guidance.

Aim 1: The only methods we will be using are queries of patient records using the Cerner and Montage programs. The Montage program allows for searching nuclear medicine records. So, an example of a search would be 'PET' and 'brown fat'.

Aim 2: We will use the VCU TelegRAM to disseminate our study information, and recruitment flyers and physician referrals at Massey Cancer Center clinics and Nuclear Medicine will be the primary source of recruitment of patients. We will also contact potential participants via email and phone based on the chart review results. The recruitment flyers will also be distributed to Massey Cancer Center and Nuclear Medicine providers. Providers will be also contacted via email. Potential participants will be identified by Massey Cancer Center and Nuclear Medicine providers who will ask the permission to introduce the study personnel to the patient for the purpose of illustrating the study and its procedures.

6. Describe any special recruitment procedures for vulnerable populations:

7. Upload all recruitment materials including ads, flyers, telephone or in-person scripts, letters, email invitations, TelegRAM announcements, and postcard reminders:

8. * Before potential participants consent to the study, will screening questions be asked or will any screening procedures/tests be done that would not otherwise be done as standard of care:
No

9. If Yes, will identifiable information about individuals be recorded during screening:

- ☐ Yes
☐ No

ID: HM20009089

View: SF - Privacy

Privacy

Privacy refers to an individual's right to control how others view, record, or obtain information about them.

Privacy is not the same as confidentiality because privacy protections apply to people, and confidentiality protections apply to data. Confidentiality protections should be described on the Data Confidentiality page of this form, not here.

1. * Describe how the research team will protect participants' privacy throughout the course of the study. Address privacy in the context of the following research activities as applicable:

- Identification of potential participants or secondary data/specimens of interest
- Recruitment and screening activities
- The informed consent process
- Conduct of the study procedures
- Data dissemination

See the help text for additional guidance.

Aim 1: As this aim is chart review, there will be no direct participant interactions that would put patient privacy at risk. We are requesting access to the minimum amount of PHI necessary. Any discussions about the data collected between the PI/Co-investigators/students will take place in a private office to ensure patient privacy.

Aim 2: Any discussion as we identify potential participants will be conducted in a private office. Recruitment will take place in a private space, such as a closed office, to ensure protection of privacy. Screening will take place in the CRSU in a private room to ensure privacy at that stage. The same will be applied to the consent process. The study will take place in the indirect calorimeter suite in the CRSU which has door to enclose the space for privacy protection. Data will be protected as outlined elsewhere and any data dissemination (poster presentations etc) will be with deidentified data only.

ID: HM20009089

View: SF - Costs to Participants

<https://irb.research.vcu.edu/irb/ResourceAdministration/Project/PrintSmartForms?Project=com.webbridge.entity.Entity%5B0ID%5BEAE13E7697DB9...> 23/38

Costs to Participants

1. Select all categories of costs that participants or their insurance companies will be responsible for:

- ☒ Participants will have no costs associated with this study
- ☐ Study related procedures that would be done under standard of care
- ☐ Study related procedures not associated with standard of care
- ☐ Administration of drugs / devices
- ☐ Study drugs or devices
- ☐ Other

2. If Other, explain:

3. * Provide details of all financial costs to the participant, other than time and transportation. Additional details regarding standard of care costs will be requested on another screen, if applicable.

Aim 1: The chart review will have interactions with or interventions involving participants, so there are no financial costs to participants.

Aim 2: No financial costs to the the participants other than time and transportation.

ID: HM20009089

View: SF - Compensation

Compensation

1. * Describe any compensation that will be provided including:

- items such as parking/transportation
- total monetary amount
- type (e.g., gift card, cash, check, merchandise, drawing, extra class credit)
- how it will be disbursed:

Aim 1: There will be no compensation

Aim 2: Staggered compensation:

\$10 via check after completion of screening visit

\$30 via check after completion of first energy recording session, plus \$5 cafeteria voucher for food purchase

\$60 via check after completion of second energy recording session, plus \$5 cafeteria voucher for food purchase

2. If compensation will be pro-rated, explain the payment schedule:

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View: SF - Risks, Discomforts, Potential Harms and Benefits

Risks, Discomforts, Potential Harms and Benefits

1. * Describe the risks of each research procedure to participants or others. For each identified risk, provide an assessment of the anticipated seriousness and likelihood of the risk. Some examples of possible risks include but are not limited to:

- Physical risks (e.g. bodily harms or discomforts, side effects, etc.)
- Psychological risks (e.g. emotional, mental, or spiritual harms or discomforts, changes to thoughts, beliefs, or behaviors, etc.)
- Research data risks (e.g. loss of confidentiality and privacy)
- Social or legal risks (e.g. impacts on relationships or reputation, legal or criminal justice actions for self or others, etc.)
- Financial risks (e.g. impacts on income, employability, or insurability, loss of services, etc.)
- Other risks (e.g. unforeseeable risks of experimental procedures, risks related to particular study designs (randomization, washout, placebo, withholding care/services, deception), etc.)

See the help text for additional guidance.

Aim 1: Since this is a chart review and there will be no interactions or interventions, the risk associated with this protocol is loss of privacy and/or confidentiality. This risk is highly unlikely, as we have established several mechanisms for protecting both privacy and confidentiality. If loss of confidentiality were to occur, the consequences of this would likely be psychological or social in nature (as opposed to physical, financial, or legal) and would not be serious, since the data collected in this study is limited to zip code, age, gender, BMI, cancer diagnosis, diabetes status, fasting glucose data, thyroid function testing results, PET image and year, and brown adipose tissue status, and MRN).

Aim 2:

Energy expenditure recording: The only risk is anxiety or discomfort due to extended period of fasting (about 8-hours overnight fasting and 4 hours fasting during the study) and limited space. Anxiety is not expected to exceed Grade 1.

Temperature modulation: Exposure to different environmental temperatures may be unpleasant. Some people are affected strongly by heat and may sweat a lot, experience low blood pressure, heavy breathing, heart palpitations, and/or dizziness. Excessive sweating is the most likely side effect but is not expected to exceed Grade 1. Palpitations would not be expected to exceed Grade 1 and are not likely as patients with serious heart conditions would not be included in this study. Dizziness is not expected to exceed Grade 2 but is not expected to be severe or result in fainting, as participants will be resting, not exercising, and can drink water and sit/lay down.

Infrared photography: There is minimal risk associated with this procedure. The pictures will be limited to the area between the jaw and the chest above the nipples.

Physiology sensors and telemetry: There is minimal risk associated with this procedure. Some individuals who are allergic or sensitive to adhesive or latex may experience skin rash (dermatitis) from the electrodes. This may be a relatively common event but not expected to exceed Grade 1.

Blood collection: There may be pain at the site where the catheter is inserted, and some bruising and very rarely infection may occur. Some people may experience temporary nausea or faint when the catheter is inserted or when they see blood. Bandages used to cover the catheter site may cause a skin rash (dermatitis) in some people who are allergic or sensitive to adhesive or latex.

Urine collection: There is minimal risk associated with this procedure. All urine will be collected in a private location.

Bio-Impedance Analysis (BIA): There is minimal risk associated with this procedure. Some individuals who are allergic or sensitive to adhesive or latex may experience skin rash (dermatitis) from the electrodes.

Quality of Life (QoL) questionnaires: There is minimal risk associated with this procedure. Participants may experience some emotional discomfort as they assess their perceived quality of life.

Thermal comfort questionnaires: There are minimal risks or discomforts associated with this procedure.

2. * Describe how the risks / harms will be minimized:

Privacy and confidentiality are discussed in other sections. Briefly, conversations regarding data and participants will be limited to private rooms with only IRB-approved personnel. Password protected files will be used for data collection, analysis, and storage.

For the overnight and study fasting, the risks are expected to be related to discomfort more than safety. In addition to the cafeteria voucher that we will give, both saltine and peanut butter crackers will be available in the unit and readily available immediately after the energy recording has finished. We will encourage participants to eat before leaving for the cafeteria or to leave campus, but we cannot force them to eat.

3. If the disclosure of any of the information obtained during the study would place the individual at risk for harm (legal, reputation, emotional etc.) and the information will be recorded so that the individual could be identified, explain the protections that will be put in place to decrease the risk of disclosure:

4. * The Code of Virginia requires that most medical personnel and all employees of institutions of higher education report suspected child/elder abuse or neglect. Is it likely investigators could discover information that would require mandatory reporting by the investigators or staff:

- ☐ Yes
☒ No

5. * Is it likely investigators could discover a participant's previously unknown condition (eg disease, suicidal thoughts, wrong paternity) or if a participant is engaging in illegal activities:

- ☒ Yes
☐ No

6. If yes, explain how and when such a discovery will be handled:

If any of the measurements that we obtain are not normal (for example, high blood sugar or high blood pressure), the participant will be informed about them. Only with the participant's permission will their primary care physician will be notified as well.

7. * Will the study's research procedures possibly identify pertinent or incidental findings that may be of importance to subjects' health?

- ☒ Yes
☐ No

8. * Describe any potential risks or harms to a community or a specific population based on study findings:

The outcome of this study has no potential to be stigmatizing or derogatory to any populations or communities.

9. * Describe criteria for withdrawing an individual participant from the study; such as safety or toxicity concerns, emotional distress, inability to comply with the protocol, etc.:

Aim 1: N/A since this is a chart review.

Aim 2: Described in detail in PRMC protocol document attached, under Section 5

10. * Summarize any pre-specified criteria for stopping or changing the study protocol due to safety concerns:

Aim 1: N/A since this is a chart review.

Aim 2: No criteria are established for changing study protocol. If safety concerns arise, as outlined in Section 5 of the PRMC protocol document, the patient can be withdrawn from the study.

11. Where appropriate, discuss provisions for ensuring necessary medical, professional, or psychological intervention in the event of adverse events to the subjects:

12. * Describe any potential for direct benefits to participants in this study:

Aim 1: N/A since this is a chart review.

Aim 2: The participant will get a personalized point estimate of their energy expenditure, which may help them with their nutritional planning to optimize health. Resting metabolic rates in the units of kCal/min will be given for the participant at both temperatures, and an example calculation to extrapolate that to a full 24 hours will be included. This letter will be sent out to the patient after their completion of the study. An example letter is now attached to this submission.

13. * Describe the scientific benefit or importance of the knowledge to be gained:

Aim 1: This chart review will provide a realistic estimate of the number of patients in the VCU health system aged 40 and older and with cancer who do and do not have brown adipose tissue visible on PET imaging. It will also allow for stratification and estimate of those numbers based on age, gender, ethnicity, BMI, weight, primary cancer diagnosis, diabetes status, medications, fasting glucose data, thyroid function testing results, and PET image visualization. This estimate is important since there is evidence implicating brown adipose tissue in cancer associated cachexia.

Aim 2: Understanding energy utilization, as well as other measures for metabolism and inflammation, in the context of adipose tissue in patients with cancer will be helpful in development of future strategies to combat cancer associated cachexia.

14. If applicable, describe alternatives (research or non-research) that are available to potential participants if they choose not to participate in this study:

15. * Indicate if this study will have a Data Safety Monitoring Board (DSMB) or a Data Safety Monitoring Plan (DSMP): [Required for all greater than minimal risk studies]

☐ DSMB

☒ DSMP

☐ No DSMB/DSMP [Note: This response is not applicable for greater than minimal risk studies]

ID: HM20009089

View: SF - DSMP Details

DSMP Details

1. * Describe your Data Safety Monitoring Plan for monitoring the study's data to ensure the safety of participants. This plan should include (but is not limited to) the following elements:

1. Who will monitor data

2. What data and/or processes will be reviewed

3. When and how frequently monitoring will occur

4. What report/documentation will be submitted to the IRB at the time of continuing reviews

See the help text for additional guidance.

The PI will be responsible for data and safety monitoring during the study. A physician not affiliated with the research proposal (Dr. Trang Le, Assistant Professor of Medicine and Pediatrics) will serve as independent compliance officer and will review every six months compliance with inclusion/exclusion criteria, gender and minority, adverse events. The results of the review will be reported to the PI and to the IRB at time of continuing review.

ID: HM20009089

View: SF - Pertinent and Incidental Findings

Pertinent and Incidental Findings

1. * Describe the possible pertinent or incidental findings stemming from research-only procedures that may be of importance to a subject's health.

As described under 'Risks, Discomforts, Potential Harms, Benefits' section, questions 5-6:

If any of the measurements that we obtain are not normal (for example, high blood sugar or high blood pressure), the participant will be informed about them. Only with the participant's permission will their primary care physician will be notified as well.

2. * Will findings be disclosed to subjects?

Yes

<https://irb.research.vcu.edu/irb/ResourceAdministration/Project/PrintSmartForms?Project=com.webbridge.entity.Entity%5B0ID%5BEAE13E7697DB9...> 26/38

1/7/2019

Print: HM20009089 - Maladaptive adipose tissue activity in cancer

3. *

Describe a communication plan addressing:

- what will be disclosed,
- to whom it will be disclosed, and
- how it will be disclosed

The participant will be informed about any incidental findings. This will take place via conversation in person with the participant in a private room. Only with the participant's permission will their primary care physician will be notified as well.

ID: HM20009089

View: SF - Consent Qualifiers

Consent Qualifiers

1. * Are you submitting your study as exempt and therefore no consent is required:

- ☐ Yes
- ☒ No

ID: HM20009089

View: SF - Consent Groups

Consent Groups

1. * List all consent groups:

Group	Types	Waivers	Roles	Roles - Other	Consent	Coercion	Decision	Re-Consent
View Charts being reviewed	None of the Above (select waiver below)	Waiver of Some or All Elements of Consent	N/A: Requesting Waiver of Consent		N/A as we are requesting waiver of consent.	N/A as we are requesting waiver of consent.	N/A as we are requesting waiver of consent.	
View Aim 2	Written/Signed Consent by Participant	No Waivers Requested	Research Nurse Principal Investigator Co/Sub-Investigator Research Coordinator		Only IRB-approved study staff will obtain consent/assent. This will take place in private rooms.	We will ensure patients that their current medical care will not be impacted whatsoever, regardless of their participation status.	One month.	

2. Upload any consent / assent documents:

ID: HM20009089

View: SF - Waiver of Some or All Elements of Consent

Waiver of Some or All Elements of Consent

Consent groups that require a waiver of some or all elements of consent:

Group	Types	Waivers	Roles	Roles - Other	Consent	Coercion	Decision	Status Change
Charts being reviewed					N/A as we are requesting waiver of consent.	N/A as we are requesting waiver of consent.	N/A as we are requesting waiver of consent.	

The basic elements of informed consent are as follows:

1. All of the following:

<https://irb.research.vcu.edu/irb/ResourceAdministration/Project/PrintSmartForms?Project=com.webbridge.entity.Entity%5B0ID%5BEAE13E7697DB9...> 27/38

- a statement that the study involves research
 - an explanation of the purposes of the research
 - an explanation of the expected duration of the participant's involvement
 - a description of the procedures to be followed
 - identification of any procedures which are experimental
2. A description of any reasonably foreseeable risks or discomforts to the participant
 3. A description of any benefits to the participant or to others which may reasonably be expected from the research
 4. A disclosure of appropriate alternative procedures or courses of treatment, if any, that might be advantageous to the participant
 5. A statement describing the extent, if any, to which confidentiality of records identifying the participant will be maintained
 6. For research involving more than minimal risk, an explanation as to whether any compensation and an explanation as to whether any medical treatments are available if injury occurs and, if so, what they consist of, or where further information may be obtained
 7. An explanation of whom to contact for answers to pertinent questions about the research and research participants' rights, and whom to contact in the event of a research-related injury to the participant
 8. A statement that participation is voluntary, refusal to participate will involve no penalty or loss of benefits to which the participant is otherwise entitled, and the participant may discontinue participation at any time without penalty or loss of benefits to which the participant is otherwise entitled
1. * Describe which of the elements of informed consent you are waiving or altering for each group listed at the top of this page:
We are requesting for all elements of informed consent to be waived for Aim 1.
2. * Will you be waiving parental permission for any of the consent groups at the top of this page:
☐ Yes
☒ No
3. * Is this study conducted by or subject to the approval of State or Local Government and designed to study, evaluate, or otherwise examine public benefit or service programs:
☐ Yes
☒ No

ID: HM20009089

View: SF - Waiver [45 CFR 46.116d] - Adults

Waiver [45 CFR 46.116d] - Adults

1. * Explain how the research involves no more than minimal risk to the participants:
The chart review involves no participant interactions or interventions, so there is no more than minimal risk to those whose charts we review.
2. * Explain how the waiver or alteration will not adversely affect the rights or welfare of the participants:
The chart review and the documentation of applicable PHI will not adversely affect the rights or welfare of any participants. Once this chart review and documentation is complete, and possible participants are contacted for recruitment, only participants who agree to enroll will continue to be contacted. Those who decline or do not sign the consent form will not be contacted again to ensure participant welfare.
3. * Explain how the research could not practicably be carried out without the waiver or alteration:
Since the chart review involves no participant interaction or interventions, we are not able to conduct this chart review and document applicable PHI without this waiver. Obtaining authorization for each chart review is not feasible in this case, as we aim to go through a large number of charts. Contact for each chart review would exceed the time available by study staff time, as we expect to review charts for an estimated 1500 patients (see Study Population section).
4. * Explain how participants will be provided with additional pertinent information after participation. If this will not be provided, explain why not:
After the chart review and documentation is complete, there will be no additional information provided to patients whose charts we reviewed.

ID: HM20009089

View: SF - Documents

Documents

1. Upload any documents that the VCU IRB will need to conduct a review of this submission.

NOTE: The delete function should only be used if an incorrect document is uploaded or added to the system AND that document has not been reviewed and approved by the IRB. Do NOT delete documents that the IRB previously reviewed and approved.

<https://irb.research.vcu.edu/irb/ResourceAdministration/Project/PrintSmartForms?Project=com.webbridge.entity.Entity%5B0ID%5BEAE13E7697DB9...> 28/38

Once you have uploaded a document to RAMS-IRB, any changes to that document (i.e. different versions of the same document) should be added to the IRB submission by using the Update button. To provide updated documents, follow these steps:

- Click the Update button located to the left of the document to be updated.
- In the Add Document window, click the Choose File or Browse button, select the file you are adding, and click on the Open button.
- Click OK to close the Add Document window, and the system will upload the revised document. RAMS-IRB will automatically provide a version number for the document.

To access previous versions of a document in RAMS-IRB you must use the History link associated with the document:

- Click the View or Update button located to the left of the document you wish to access.
- In the Add/View Document window, click the "History" hyperlink located to the right of the file name.
- A separate window will open that shows all versions of the document that have been added to RAMS-IRB. Click on any file name to download and view the document.

Document Name	Document	Version	Date Modified	Uploaded By	Type	Approved
View CV_Stamatouli	StamatouliCVsigned.pdf	0.01	11/20/2018 8:33 AM	Janina Pearce	CV/Biosketch	Not Applicable
View Consent_CoC	2018.07 Consent.pdf	0.12	8/8/2018 10:35 AM	Janina Pearce	Consent/Assent/Information Sheet	Yes
View Protocol_PRMC	2018.07 PRMC.docx	0.06	7/24/2018 12:12 PM	Janina Pearce	Research Protocol	Yes
View Email script	2018.04 Email contact.docx	0.01	4/30/2018 8:15 AM	Janina Pearce	Recruitment/Advertising	Yes
View Phone script	2018.04 Phone contact.docx	0.01	4/30/2018 8:15 AM	Janina Pearce	Recruitment/Advertising	Yes
View TelegRAM	2018.04 TelegRAM.docx	0.01	4/30/2018 8:15 AM	Janina Pearce	Recruitment/Advertising	Yes
View StudyFlyerRecruitment	2018.04 Recruitment Flyer.docx	0.02	4/30/2018 8:12 AM	Janina Pearce	Recruitment/Advertising	Yes
View EnergyExpenditureLetter_Example	2018.04 ParticipantResults.docx	0.01	4/25/2018 7:28 AM	Janina Pearce	Other	Yes
View MasseyPilotFunding	19. Funding Notice Celi_Chen_Del Fabbro.pdf	0.01	2/23/2018 11:53 AM	Janina Pearce	Funding Proposal	Not Applicable
View Questionnaire_ThermalComfort	Thermal Comfort Questionnaire.pdf	0.01	2/23/2018 8:45 AM	Janina Pearce	Research Measure	Yes
View Questionnaire_QualityOfLife	QoL FAACT Questionnaire.pdf	0.01	2/23/2018 8:45 AM	Janina Pearce	Research Measure	Yes
View Chen CV	SChenCV.pdf	0.01	2/20/2018 10:27 AM	Janina Pearce	CV/Biosketch	Not Applicable
View MCC_PRMC_ProspectiveStudy Letter	2018-01-08_MCC-17-13470_PRMC Approval Letter.pdf	0.01	2/20/2018 10:26 AM	Janina Pearce	Ancillary Committee Approval	Not Applicable
View MCC_PRMC_ChartReview Letter	2017-01-17_MCC-17-13218_PRMC Review.pdf	0.01	1/25/2017 8:51 PM	Francesco Celi	Ancillary Committee Approval	Not Applicable
View References for Research Description	References for Research Description.docx	0.01	11/11/2016 9:13 AM	Janina Pearce	Research Protocol	Not Applicable
View Del Fabbro CV	Del Fabbro CV 2016 oct.pdf	0.01	11/2/2016 9:36 AM	Janina Pearce	CV/Biosketch	Not Applicable
View Celi CV	FS Celi CV August_2016_VCU.doc	0.01	11/2/2016 9:36 AM	Janina Pearce	CV/Biosketch	Yes
View Vaitkus Biosketch	Vaitkus_Biosketch2.pdf	0.01	10/31/2016 12:36 PM	Janina Pearce	CV/Biosketch	Yes

ID: HM20009089

View: SF - Protocol Complete

Section Complete:

SUMMARY

<https://irb.research.vcu.edu/irb/ResourceAdministration/Project/PrintSmartForms?Project=com.webbridge.entity.Entity%5B0ID%5BEAE13E7697DB9...> 29/38

End of Application:
IRB HUMAN SUBJECTS STUDY

Click Continue Below

ID: HM20009089

View: Personnel

Personnel

1. * Name:

Francesco Celli

2. * Is this individual a 'COI Investigator'?

- ☒ Yes
☐ No

3. * Roles:

- | | |
|-------------------------------------|---|
| <input checked="" type="checkbox"/> | Principal Investigator |
| <input type="checkbox"/> | Co/Sub-Investigator |
| <input type="checkbox"/> | Medical or Psychological Responsible Investigator |
| <input type="checkbox"/> | Student Investigator |
| <input type="checkbox"/> | Research Coordinator |
| <input type="checkbox"/> | Research Nurse |
| <input type="checkbox"/> | Consultant |
| <input type="checkbox"/> | Research Assistant |
| <input type="checkbox"/> | Pharmacist |
| <input type="checkbox"/> | Statistician |
| <input type="checkbox"/> | Regulatory Coordinator |
| <input type="checkbox"/> | Trainee/Student |
| <input type="checkbox"/> | Other |

4. If other role is selected, explain:

5. * Study related responsibilities:

- | | |
|-------------------------------------|--------------------------------------|
| <input checked="" type="checkbox"/> | Study Design |
| <input checked="" type="checkbox"/> | Data Collection - Lab |
| <input checked="" type="checkbox"/> | Data Collection - Clinical |
| <input checked="" type="checkbox"/> | Data Collection - Interviews/Surveys |
| <input checked="" type="checkbox"/> | Data Collection - Direct Observation |
| <input type="checkbox"/> | Clinical Services |
| <input type="checkbox"/> | Intervention Services |
| <input checked="" type="checkbox"/> | Data Entry |
| <input checked="" type="checkbox"/> | Data Coding |
| <input checked="" type="checkbox"/> | Data Management |
| <input checked="" type="checkbox"/> | Data Analysis |
| <input checked="" type="checkbox"/> | Project Coordination |
| <input checked="" type="checkbox"/> | Participant Identification |
| <input checked="" type="checkbox"/> | Participant Recruitment |
| <input checked="" type="checkbox"/> | Participant Consent |
| <input type="checkbox"/> | Regulatory Management |

☐ Other

6. If other responsibility is selected, explain:

7. * The PI certifies that if this individual will conduct any clinical activities as part of this study, the individual is appropriately credentialed and privileged to practice within the institution where the research will be conducted:
Yes

8. * Qualifications to carry out study related responsibilities: (you may select multiple answers)

☒ Education and/or Professional Preparation

☒ Experience - Research

☒ Experience - Clinical

☒ Experience - Related Skills

☐ Trainee

☐ Student

☐ Other

9. If other qualification is selected, explain:

10. Additional or Emergency Phone:

ID: HM20009089

View: Personnel

Personnel

1. * Name:

Janina Pearce

2. * Is this individual a 'COI Investigator'?

☒ Yes

☐ No

3. * Roles:

☐ Principal Investigator

☐ Co/Sub-Investigator

☐ Medical or Psychological Responsible Investigator

☐ Student Investigator

☐ Research Coordinator

☐ Research Nurse

☐ Consultant

☐ Research Assistant

☐ Pharmacist

☐ Statistician

☐ Regulatory Coordinator

☒ Trainee/Student

☐ Other

4. If other role is selected, explain:

5. * Study related responsibilities:

☒ Study Design

☒ Data Collection - Lab

☒ Data Collection - Clinical

<input checked="" type="checkbox"/>	Data Collection - Interviews/Surveys
<input checked="" type="checkbox"/>	Data Collection - Direct Observation
<input type="checkbox"/>	Clinical Services
<input type="checkbox"/>	Intervention Services
<input checked="" type="checkbox"/>	Data Entry
<input checked="" type="checkbox"/>	Data Coding
<input checked="" type="checkbox"/>	Data Management
<input checked="" type="checkbox"/>	Data Analysis
<input checked="" type="checkbox"/>	Project Coordination
<input checked="" type="checkbox"/>	Participant Identification
<input checked="" type="checkbox"/>	Participant Recruitment
<input checked="" type="checkbox"/>	Participant Consent
<input type="checkbox"/>	Regulatory Management
<input type="checkbox"/>	Other

6. If other responsibility is selected, explain:

7. * The PI certifies that if this individual will conduct any clinical activities as part of this study, the individual is appropriately credentialed and privileged to practice within the institution where the research will be conducted:
Individual has no clinical responsibilities

8. * Qualifications to carry out study related responsibilities: (you may select multiple answers)

<input type="checkbox"/>	Education and/or Professional Preparation
<input type="checkbox"/>	Experience - Research
<input type="checkbox"/>	Experience - Clinical
<input type="checkbox"/>	Experience - Related Skills
<input type="checkbox"/>	Trainee
<input checked="" type="checkbox"/>	Student
<input type="checkbox"/>	Other

9. If other qualification is selected, explain:

10. Additional or Emergency Phone:

ID: HM20009089

View: Personnel

Personnel

1. * Name:

Egidio Del Fabbro

2. * Is this Individual a 'COI Investigator'?

- ☒ Yes
☐ No

3. * Roles:

<input type="checkbox"/>	Principal Investigator
<input checked="" type="checkbox"/>	Co/Sub-Investigator
<input type="checkbox"/>	Medical or Psychological Responsible Investigator
<input type="checkbox"/>	Student Investigator
<input type="checkbox"/>	Research Coordinator
<input type="checkbox"/>	Research Nurse

<input type="checkbox"/>	Consultant
<input type="checkbox"/>	Research Assistant
<input type="checkbox"/>	Pharmacist
<input type="checkbox"/>	Statistician
<input type="checkbox"/>	Regulatory Coordinator
<input type="checkbox"/>	Trainee/Student
<input type="checkbox"/>	Other

4. If other role is selected, explain:

5. * Study related responsibilities:

<input checked="" type="checkbox"/>	Study Design
<input checked="" type="checkbox"/>	Data Collection - Lab
<input checked="" type="checkbox"/>	Data Collection - Clinical
<input checked="" type="checkbox"/>	Data Collection - Interviews/Surveys
<input checked="" type="checkbox"/>	Data Collection - Direct Observation
<input type="checkbox"/>	Clinical Services
<input type="checkbox"/>	Intervention Services
<input checked="" type="checkbox"/>	Data Entry
<input checked="" type="checkbox"/>	Data Coding
<input checked="" type="checkbox"/>	Data Management
<input checked="" type="checkbox"/>	Data Analysis
<input checked="" type="checkbox"/>	Project Coordination
<input checked="" type="checkbox"/>	Participant Identification
<input checked="" type="checkbox"/>	Participant Recruitment
<input checked="" type="checkbox"/>	Participant Consent
<input type="checkbox"/>	Regulatory Management
<input type="checkbox"/>	Other

6. If other responsibility is selected, explain:

7. * The PI certifies that if this individual will conduct any clinical activities as part of this study, the individual is appropriately credentialed and privileged to practice within the institution where the research will be conducted:
Yes

8. * Qualifications to carry out study related responsibilities: (you may select multiple answers)

<input checked="" type="checkbox"/>	Education and/or Professional Preparation
<input checked="" type="checkbox"/>	Experience - Research
<input checked="" type="checkbox"/>	Experience - Clinical
<input checked="" type="checkbox"/>	Experience - Related Skills
<input type="checkbox"/>	Trainee
<input type="checkbox"/>	Student
<input type="checkbox"/>	Other

9. If other qualification is selected, explain:

10. Additional or Emergency Phone:

ID: HM20009089

View: Personnel

Personnel

<https://irb.research.vcu.edu/irb/ResourceAdministration/Project/PrintSmartForms?Project=com.webbridge.entity.Entity%5B0ID%5BEAE13E7697DB9...> 33/38

1. * Name:

Shanshan Chen

2. * Is this Individual a 'COI Investigator'?☒ Yes☐ No**3. * Roles:**

- ☐ Principal Investigator
- ☒ **Co/Sub-Investigator**
- ☐ Medical or Psychological Responsible Investigator
- ☐ Student Investigator
- ☐ Research Coordinator
- ☐ Research Nurse
- ☐ Consultant
- ☐ Research Assistant
- ☐ Pharmacist
- ☒ **Statistician**
- ☐ Regulatory Coordinator
- ☐ Trainee/Student
- ☐ Other

4. If other role is selected, explain:**5. * Study related responsibilities:**

- ☒ **Study Design**
- ☒ **Data Collection - Lab**
- ☒ **Data Collection - Clinical**
- ☒ **Data Collection - Interviews/Surveys**
- ☒ **Data Collection - Direct Observation**
- ☐ Clinical Services
- ☐ Intervention Services
- ☒ **Data Entry**
- ☒ **Data Coding**
- ☒ **Data Management**
- ☒ **Data Analysis**
- ☒ **Project Coordination**
- ☒ **Participant Identification**
- ☒ **Participant Recruitment**
- ☒ **Participant Consent**
- ☐ Regulatory Management
- ☐ Other

6. If other responsibility is selected, explain:**7. * The PI certifies that If this Individual will conduct any clinical activities as part of this study, the Individual is appropriately credentialed and privileged to practice within the institution where the research will be conducted:**
Individual has no clinical responsibilities**8. * Qualifications to carry out study related responsibilities: (you may select multiple answers)**

- ☒ **Education and/or Professional Preparation**
- ☒ **Experience - Research**
- ☒ **Experience - Clinical**
- ☒ **Experience - Related Skills**

- ☐ Trainee
- ☐ Student
- ☐ Other

9. If other qualification is selected, explain:

10. Additional or Emergency Phone:

ID: HM20009089

View: Personnel

Personnel

1. * Name:

Tamas Gal

2. * Is this individual a 'COI Investigator'?

- ☒ Yes
- ☐ No

3. * Roles:

- ☐ Principal Investigator
- ☐ Co/Sub-Investigator
- ☐ Medical or Psychological Responsible Investigator
- ☐ Student Investigator
- ☐ Research Coordinator
- ☐ Research Nurse
- ☒ Consultant
- ☐ Research Assistant
- ☐ Pharmacist
- ☒ Statistician
- ☐ Regulatory Coordinator
- ☐ Trainee/Student
- ☐ Other

4. If other role is selected, explain:

5. * Study related responsibilities:

- ☐ Study Design
- ☐ Data Collection - Lab
- ☒ Data Collection - Clinical
- ☐ Data Collection - Interviews/Surveys
- ☐ Data Collection - Direct Observation
- ☐ Clinical Services
- ☐ Intervention Services
- ☒ Data Entry
- ☒ Data Coding
- ☒ Data Management
- ☒ Data Analysis
- ☐ Project Coordination
- ☐ Participant Identification
- ☐ Participant Recruitment

- ☐ Participant Consent
- ☐ Regulatory Management
- ☐ Other

6. If other responsibility is selected, explain:

7. * The PI certifies that if this individual will conduct any clinical activities as part of this study, the individual is appropriately credentialed and privileged to practice within the institution where the research will be conducted:
Individual has no clinical responsibilities

8. * Qualifications to carry out study related responsibilities: (you may select multiple answers)

- ☒ Education and/or Professional Preparation
- ☒ Experience - Research
- ☒ Experience - Clinical
- ☒ Experience - Related Skills
- ☐ Trainee
- ☐ Student
- ☐ Other

9. If other qualification is selected, explain:

10. Additional or Emergency Phone:

ID: HM20009089

View: Personnel

Personnel

1. * Name:

Martin Charron

2. * Is this individual a 'COI Investigator'?

- ☒ Yes
- ☐ No

3. * Roles:

- ☐ Principal Investigator
- ☐ Co/Sub-Investigator
- ☐ Medical or Psychological Responsible Investigator
- ☐ Student Investigator
- ☐ Research Coordinator
- ☐ Research Nurse
- ☒ Consultant
- ☐ Research Assistant
- ☐ Pharmacist
- ☐ Statistician
- ☐ Regulatory Coordinator
- ☐ Trainee/Student
- ☐ Other

4. If other role is selected, explain:

5. * Study related responsibilities:

- ☐ Study Design

<input type="checkbox"/>	Data Collection - Lab
<input checked="" type="checkbox"/>	Data Collection - Clinical
<input type="checkbox"/>	Data Collection - Interviews/Surveys
<input type="checkbox"/>	Data Collection - Direct Observation
<input type="checkbox"/>	Clinical Services
<input type="checkbox"/>	Intervention Services
<input checked="" type="checkbox"/>	Data Entry
<input checked="" type="checkbox"/>	Data Coding
<input checked="" type="checkbox"/>	Data Management
<input checked="" type="checkbox"/>	Data Analysis
<input type="checkbox"/>	Project Coordination
<input checked="" type="checkbox"/>	Participant Identification
<input type="checkbox"/>	Participant Recruitment
<input type="checkbox"/>	Participant Consent
<input type="checkbox"/>	Regulatory Management
<input type="checkbox"/>	Other

6. If other responsibility is selected, explain:

7. * The PI certifies that if this individual will conduct any clinical activities as part of this study, the individual is appropriately credentialed and privileged to practice within the institution where the research will be conducted:
Yes

8. * Qualifications to carry out study related responsibilities: (you may select multiple answers)

<input checked="" type="checkbox"/>	Education and/or Professional Preparation
<input checked="" type="checkbox"/>	Experience - Research
<input checked="" type="checkbox"/>	Experience - Clinical
<input checked="" type="checkbox"/>	Experience - Related Skills
<input type="checkbox"/>	Trainee
<input type="checkbox"/>	Student
<input type="checkbox"/>	Other

9. If other qualification is selected, explain:

10. Additional or Emergency Phone:

ID: HM20009089

View: Personnel

Personnel

1. * Name:

Angeliki Stamatouli

2. * Is this individual a 'COI Investigator'?

- ☒ Yes
☐ No

3. * Roles:

<input type="checkbox"/>	Principal Investigator
<input checked="" type="checkbox"/>	Co/Sub-Investigator
<input type="checkbox"/>	Medical or Psychological Responsible Investigator
<input type="checkbox"/>	Student Investigator

- ☐ Research Coordinator
- ☐ Research Nurse
- ☐ Consultant
- ☐ Research Assistant
- ☐ Pharmacist
- ☐ Statistician
- ☐ Regulatory Coordinator
- ☐ Trainee/Student
- ☐ Other

4. If other role is selected, explain:

5. * Study related responsibilities:

- ☐ Study Design
- ☒ Data Collection - Lab
- ☒ Data Collection - Clinical
- ☒ Data Collection - Interviews/Surveys
- ☒ Data Collection - Direct Observation
- ☒ Clinical Services
- ☒ Intervention Services
- ☒ Data Entry
- ☐ Data Coding
- ☐ Data Management
- ☒ Data Analysis
- ☒ Project Coordination
- ☒ Participant Identification
- ☒ Participant Recruitment
- ☒ Participant Consent
- ☐ Regulatory Management
- ☐ Other

6. If other responsibility is selected, explain:

7. * The PI certifies that If this individual will conduct any clinical activities as part of this study, the individual is appropriately credentialed and privileged to practice within the institution where the research will be conducted:
Yes

8. * Qualifications to carry out study related responsibilities: (you may select multiple answers)

- ☒ Education and/or Professional Preparation
- ☒ Experience - Research
- ☒ Experience - Clinical
- ☐ Experience - Related Skills
- ☐ Trainee
- ☐ Student
- ☐ Other

9. If other qualification is selected, explain:

10. Additional or Emergency Phone:

Appendix B

Human Subjects Research Protocol

Title: Maladaptive adipose tissue activity in cancer

MCC Protocol #: MCC-17-13470

Principal Investigator:

Francesco S. Celi, MD, MHSc
Division of Endocrinology Diabetes and Metabolism
Department of Internal Medicine
1101 East Marshall Street, 7th floor
Richmond, Virginia 23298
(804) 828-9696
Francesco.celi@vcuhealth.org

Subinvestigator/Responsible Investigator:

Janina Vaitkus Pearce, MD-PhD Student
Egidio Del Fabbro, MD
Angeliki Stamatouli, MD
Martin Charron, MD
Shanshan Chen, PhD
Tamas Gal, PhD, MS

Biostatistician:

Shanshan Chen, PhD
Tamas Gal, PhD, MS

Funding Sponsor:

Virginia Commonwealth University Massey
Cancer Center Pilot Project

Responsible Research Nurse:

Joyce R. Ruddley RN, BSN, CCRP

Investigational Agent(s):

Whole-room indirect calorimeters

Version #: 6

Version Date: 01/07/2019

PROTOCOL SUMMARY

Title: Maladaptive brown adipose tissue activity in cancer

Protocol Number:

IND Sponsor: Not applicable.

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Clinical Trial Phase: Pilot Study

Study Disease: Cancer-associated cachexia (CAC)

Main Eligibility Criteria:
≥ 40 years of age
Have active cancer
Have had a PET/CT scan within the past 36 months at time of contact
Ability to understand and the willingness to sign a written informed consent document.

Primary Objectives: To characterize and measure the difference in energy metabolism profiles of patients with cancer with and without evidence of Brown Adipose Tissue (BAT) activation both at room temperature and following exposure to warm temperature.

Secondary Objectives:	To measure the differences in perceived quality of life, perceived thermal comfort, serum values of glucose, lipid, free fatty acids, and exosomes, and urine values of creatinine, cortisol, electrolytes, catecholamines, and metanephrines of patients with cancer with and without evidence of BAT activation.
Endpoints:	<p>(Primary) Difference in resting energy expenditure between BAT-positive and BAT-negative patients with cancer.</p> <p>(Primary) Difference in energy expenditure between room temperature and response to warm exposure (Δ energy expenditure) in BAT-positive and BAT-negative cancer patients.</p> <p>(Secondary) Difference in perceived quality of life between BAT-positive and BAT-negative patients with cancer.</p> <p>(Secondary) Difference in perceived thermal comfort between BAT-positive and BAT-negative patients with cancer.</p> <p>(Secondary) Difference in serum values of glucose, lipids, free fatty acid and exosomes between BAT-positive and BAT-negative patients with cancer.</p> <p>(Secondary) Difference in urine values of creatinine, cortisol, electrolytes, catecholamines, and metanephrines between BAT-positive and BAT-negative patients with cancer.</p>
Study Design:	<p>Cancer-associated cachexia is a common and complex condition in patients with many types of cancer, characterized by the loss of skeletal (muscle) and/or adipose tissue (fat) mass that negatively affects patient quality of life. Its presence limits the use of anti-tumor therapies and is associated with poor outcomes. Cancer-associated cachexia is ultimately caused by a sustained negative energy balance due to decreased energy intake and/or increased energy expenditure.</p> <p>Several studies suggest that patients affected by cancer, especially in advanced stages, have an increase in BAT, a specialized form of adipose tissue that has the unique capability of burning energy and generating heat. BAT activity can be seen in the cervical (neck and shoulders) region in PET/CT studies, which are routinely performed in patients with cancer to monitor tumor size and progression. Some studies suggest that inflammation associated with cancer plays a role in the activation and expansion of BAT in experimental models of cancer, but the clinical importance of BAT in patients with cancer is unknown.</p> <p>We hypothesize that blocking the activation of BAT in patients with cancer will result in a decrease in energy expenditure. This could potentially halt, or even reverse, the weight loss seen in cancer-associated cachexia, ultimately improving patients' quality of life and increase their ability to receive appropriate anti-tumor treatments. We will recruit patients with cancer and BAT activation to compare their energy expenditure with the energy expenditure</p>

	<p>of age-, sex-, BMI-, and cancer type-matched patients with no BAT activation at room temperature. The working hypothesis is that patients with evidence of BAT activity at room temperature will exhibit, compared to BAT-negative controls, an increase in energy expenditure which could contribute to the development and maintenance of cancer-associated cachexia.</p> <p>We will then assess if exposure to warm temperature, a known intervention to quench brown fat activation, is sufficient to decrease the energy expenditure in cancer patients with evidence of BAT activation.</p> <p>Other data that will be collected include: a quality of life questionnaire, thermal comfort questionnaires, infrared photography, blood collection, urine collection.</p> <p>The results of these studies will provide strong and convincing preliminary data for a larger NIH grant proposal that will further investigate this and other novel interventions aimed at decreasing brown fat activation in patients with cancer, to eventually improve their outcomes and quality of life.</p>
Study Agent/ Intervention Description:	Whole-room indirect calorimeters. The indirect calorimeter technique allows for non-invasive real-time recording of energy expenditure and respiratory quotient (a proxy for substrate utilization) by measuring oxygen consumption and carbon dioxide production.
Number of Subjects:	36
Subject Participation Duration:	Up to one month post screening visit
Estimated Time to Complete Enrollment:	2 years
Statistical Methodology:	<p>One-sided t-test for primary endpoint: Difference in resting energy expenditure between BAT-positive and BAT-negative patients with cancer.</p> <p>Two-sided t-test for primary endpoint: Difference in energy expenditure between room temperature and response to warm exposure (Δ energy expenditure) in BAT-positive and BAT-negative cancer patients.</p> <p>Mann-Whitney U-tests for secondary endpoints: Difference in perceived quality of life between BAT-positive and BAT-negative patients with cancer, and Difference in perceived thermal comfort between BAT-positive and BAT-negative patients with cancer.</p> <p>Two sample, two-sided t-tests for components within secondary endpoints: Difference in serum values of glucose, lipids, free fatty acid and exosomes between BAT-positive and BAT-negative patients with cancer, and Difference in urine values of creatinine,</p>

cortisol, electrolytes, catecholamines, and metanephrines between
BAT-positive and BAT-negative patients with cancer.

SCHEMA

Table 1: Schedule of Events.

Assessment	Screening	Visit 2	Visit 3
Informed Consent	X		
Confirm Inclusion/Exclusion	X		
Medical History	X		
Physical Examination	X		
Weight	X		
Vital Signs	X		
Bio-impedance analysis	X		
QOL questionnaire	X		
Resting Energy Expenditure		X	X
Infrared Photography		X	X
Physiology Sensors and Telemetry		X	X
Blood Collection		X	X
Urine Collection		X	X
Thermal Comfort Questionnaires		X	X

REVISION HISTORY

Revision history is presented in reverse order so that the information pertaining to the most current version of the protocol is presented first in this section.

Version 6, Version Date 1/7/2019: Addition of Dr. Angeliki Stamatouli, initiated by investigators

Version 5, Version Date 7/24/2018: Minor changes made based on IRB review

Version 4, Version Date 06/2018: Minor changes made initiated by investigators

Version 3, Version Date 04/2018: Minor changes made based on IRB review

Version 2, Version Date 01/08/2018: Minor changes made based on Reviewer Comments

Version 1, Version Date 12/08/2017: Initial submission of the protocol

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LIST OF ABBREVIATIONS

Abbreviations	Description of abbreviations
AE	Adverse Event
BAT	Brown adipose tissue
BIA	Bio-electrical Impedance Analysis
BMI	Body Mass Index
CRF	Case Report Form
CAC	Cancer-associated cachexia
CRSU	Clinical Research Service Unit
CT	Computerized Tomography
ECG	Electrocardiogram
EE	Energy Expenditure
EMG	Electromyography
FAACT	Functional Assessment of Anorexia-Cachexia Therapy
GWAS	Genome-wide association study
IRB	Institutional Review Board
MPC	Monroe Park Campus
PET	Positron Emission Tomography
PG-SGA	Scored Patient-Generated Subjective Global Assessment
SAE	Serious Adverse Event
UCP1	Uncoupling protein 1
UP	Unanticipated Problem
WAT	White adipose tissue
QOL	Quality of Life

1 BACKGROUND

1.1 Cancer-Associated Cachexia

Cancer-associated cachexia (CAC), a “multifactorial syndrome defined by an ongoing loss of skeletal muscle mass (with or without loss of fat mass)”¹, is a major contributor to the decrease in quality of life and life expectancy in patients affected by cancer. CAC represents an independent poor prognostic factor for survival, affecting up to 80% of patients with advanced cancer² and contributing to upwards of 20% of cancer-related deaths³. There is currently no specific treatment for CAC, and various trials have been inconclusive or limited in achieving clinically meaningful improvements. There is a clear need to develop novel strategies to treat or ameliorate this condition. A catabolic state is the hallmark of CAC, and the sustained negative energy balance was historically thought to be secondary to poor dietary intake due to anorexia, mucositis, dysgeusia, or malabsorption^{4,5}. However, evidence now suggests that increased energy expenditure is a key contributor to the negative energy balance seen in CAC. Several recent studies indicated that an increase in energy expenditure may contribute to the establishment and maintenance of CAC⁶. This could be due to the metabolic demands of the tumor or due to the increase energy metabolism of the host, possibly driven by the inflammatory state associated with malignancy. Interestingly, interleukin-6 (IL-6), whose tissue and circulating levels are increased in patients with malignancy, may contribute to the increase in energy expenditure by inducing the browning of white adipose tissue (WAT)⁷. Indeed, an increase in the protein expression of uncoupling protein 1 (UCP1), the hallmark of for brown fat metabolic activity^{8,9}, was observed in white adipose tissue of mice injected with an IL-6-proficient C26 carcinoma cell line compared to mice injected with an IL-6-deficient C26 carcinoma cell line¹⁰, indicating browning of WAT to beige adipocytes (where beige adipocytes are white adipocytes that have shifted to the brown adipocyte phenotype and express UCP1), ultimately contributing to increased energy expenditure⁷. Brown Adipose Tissue (BAT) has the unique ability of dissipating chemical energy in the form of glucose and free fatty acids into heat under the stimulation of the beta-3 adrenergic pathway and can contribute to a significant increase in energy metabolism. Of note, the rediscovery of BAT in adult humans has been driven by the availability of PET/CT imaging. While originally considered a nuisance by radiologist in studies of the torso-cervical areas, BAT is now an additional finding that may confer clinical significance. Classically, BAT activity is driven by cold exposure¹¹ and the prevalence of BAT is greater in young and lean individuals, with negligible prevalence in individuals above 40 years old. Pre-treatment with beta blockers, or exposure to warm temperature have been developed as effective strategies to minimize BAT activation^{12,13}. Our group has demonstrated that activation of BAT is sufficient to generate a significant increase in energy expenditure, which could result in clinically relevant weight loss¹⁴. Recent observations from a retrospective chart review (*see preliminary data*) indicate that BAT activity is present in a significant number of adult patients over 40 years of age treated at Massey Cancer Center. This is important because, as mentioned previously, the prevalence of BAT activity in healthy individuals over the age of 40 is negligible, indicating that these cancer patients are likely to have maladaptive BAT expansion and activation driving inappropriately-increased energy expenditure. The premise of this proposal is that the tumor microenvironment stimulates maladaptive expansion and activation of BAT at room temperature, resulting in sustained increase in energy expenditure, which then contributes to the net-negative energy imbalance promoting CAC. Our working hypothesis is that inhibition of maladaptive BAT activation may result in restoration of the energy balance, ultimately attenuating CAC and possibly improving prognosis, life expectancy, and quality of life. To assess this novel hypothesis, we have

developed a set of research aims that will allow us to collect invaluable preliminary data to use in an extramural grant application aimed toward ameliorating CAC by addressing an underappreciated, yet easily targetable, pathway. **Aim 1:** Assessment of resting energy expenditure at room temperature (24°C), by whole room indirect calorimetry in 1) patients with cancer with PET/CT scans positive for BAT, and 2) age-, sex-, BMI-, and primary malignancy-matched patients with cancer with BAT negative PET/CT scans. **Aim 2:** Inhibition of maladaptive BAT activity in patients affected by malignancy. We will assess the effects of environmental temperature modulation (increasing temperature to 27°) on resting energy expenditure in patients with PET/CT scans positive for BAT, and matched BAT negative controls by whole room indirect calorimetry.

Preliminary Data: We have conducted two types of preliminary experiments to determine whether tumor microenvironment induces changes in adipose tissue that increase energy expenditure. **a. Morphological studies.** We performed immunohistochemistry for UCP1 staining on breast cancer tissue in collaboration with the VCU Tissue and Data Analysis and Acquisition Core (TDAAC). We chose breast cancer as our experimental model because of it is unique in its location in a tissue naturally enriched in adipose tissue, enabling us to assess the hypothesis that the tumor microenvironment promotes browning of the surrounding adipose tissue. The preliminary data qualitatively show that the adipose tissue

at the tumor margins is enriched in UCP1 when compared to control breast tissue (Figure 1). In order to further characterize this finding, we next replicated the experiment in a murine model of spontaneous breast cancer, and performed a quantitative analysis of the intensity of UCP1 staining (Figure 2). Figure 2D clearly indicates that the UCP1 protein expression is highest at the interface tumor-adipose tissue and progressively decreases as the distance from the tumor increases, capturing the inverse power law between the proximity to tumor margins and intensity of UCP1 expression. These data strongly support the hypothesis that the tumor microenvironment promotes the browning of adipose tissue surrounding or interfacing the tumor. **b. Diagnostic imaging.**

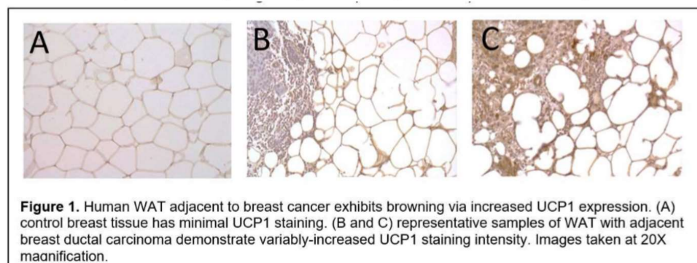


Figure 1. Human WAT adjacent to breast cancer exhibits browning via increased UCP1 expression. (A) control breast tissue has minimal UCP1 staining. (B and C) representative samples of WAT with adjacent breast ductal carcinoma demonstrate variably-increased UCP1 staining intensity. Images taken at 20X magnification.

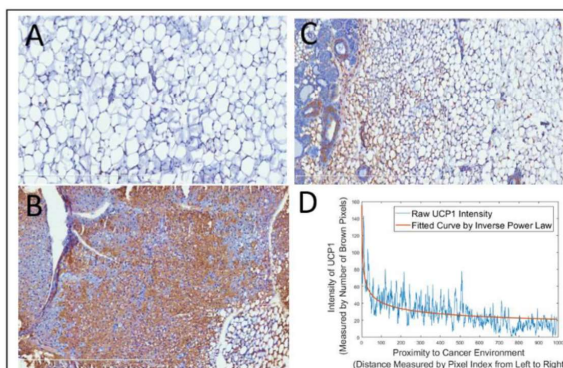
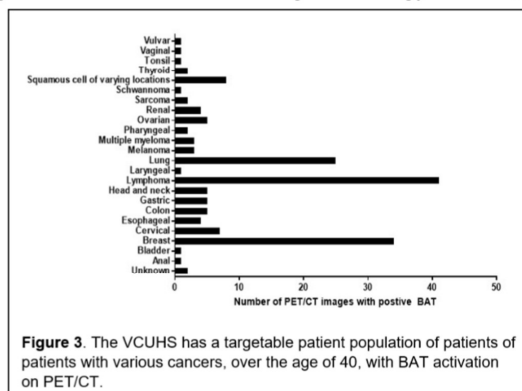


Figure 2. WAT adjacent to murine mammary tumor resembles beige adipose tissue. (A) ovarian WAT used as negative control has minimal UCP1 staining and large lipid droplets. (B) Intrascapular BAT used as positive control has high UCP1 staining and small, multilocular lipid droplets. (C) Inguinal WAT adjacent to mammary tumor appears to have smaller lipid droplets and increased UCP1 staining intensity compared to ovarian WAT, resembling the beige phenotype. (D) Analysis of the number of brown pixels, proxy for UCP1 staining intensity, demonstrating maximal staining at the tumor-adipose interface and decreasing with distance from the tumor. All images taken from one representative mouse at 10X magnification.

We performed an IRB-approved chart review (HM20009089, MCC-17-13218) to identify patients ages 40 and older with any type of primary cancer and BAT activity noted on their PET/CT imaging results using the Cerner database and Montage™ natural language search tool. We used keywords “Brown fat” and “brown adipose tissue” to locate images with BAT. The results indicate that over a period of 10 years, 164 unique individuals had evidence of BAT activity as incidental finding during PET/CT scans. The majority of cases were clustered in patients with leukemia/lymphoma, breast, and lung cancer (Figure 3), suggesting that BAT activation is prevalent in the population served by Massey Cancer Center. Note that the methodology employed in this search likely underestimates the actual prevalence of BAT because of the limitation of the natural language search. Importantly, in the general population BAT activity is inversely related with age, and negligible beyond 40 years. Hence the presence of BAT activity in the study population is clearly attributable to the underlying malignancy. Collectively, our preliminary data support the hypothesis that maladaptive expansion and activation of thermogenic (beige and brown) adipose tissue may contribute in some cancer patients to generate and sustain a net negative energy balance, promoting the development of CAC. Furthermore, within the VCUHS and Massey Cancer Center population, there is a sufficient number of patients who meet our criteria for participating in a pilot study to assess the effects of BAT activation on energy expenditure. With our unique resources and expertise, (*described below*) we have the tools and skills to successfully conduct an innovative project with the potential of developing a new and safe therapeutic modality to prevent and treat CAC.



1.2 Whole-room indirect calorimeters

The indirect calorimeter instruments and techniques allow for non-invasive real-time recording of energy expenditure and respiratory quotient by measuring oxygen consumption and carbon dioxide production¹⁵. In comparison with alternate forms of measuring energy expenditure (the historically-used metabolic carts and portable calorimeters), the whole room calorimeters overcome the issues of: 1) Low accuracy due to environmental interference (relative humidity, oxygen and carbon dioxide concentration in the testing room, presence of study personnel); 2) Inconvenience of using a face mask or a canopy hood which limit the duration of the observations and the flexibility of conducting tests which require additional sensors or manipulations. Conversely, whole room calorimeters, by allowing free movement of the subject and through controlling and monitoring environmental factors (e.g. temperature, gas concentration in the inflow air), overcome the limitations of the metabolic carts and portable calorimeters. Traditionally, room calorimeters were challenged by the long equilibration period and limited ability of capturing dynamic interventions with time resolution of less than 30 minutes¹⁶. At VCU, we have recently built and validated a fast-response whole-room calorimeter suite, which allows a fast equilibration period (10

minutes) and recording dynamic changes within 10-15 minutes, favorably comparable to the metabolic cart methods. Additionally, the environmental temperature of our systems can be modulated between 15°C - 30°C (59°F - 86°F) while maintaining an accurate reading. Our room calorimeters are equipped with airtight ports for food administration, TV sets, a toilet, and Wi-Fi connectivity, which enable more comfortable and longer studies, as well as direct observation of acute (minutes to hours) effects of pharmacologic interventions on energy expenditure and substrate utilization. The flexibility of the VCU whole-room indirect calorimeter system allows for continuous and noninvasive monitoring of energy expenditure, and other parameters such as heart rate and skin temperature, while the volunteer is in a comfortable environment. In previous experiments, using a less sensitive model of a whole room calorimeter, we have demonstrated that compared to room temperature (24°C or 75°F), a 12-hour moderate cold exposure (19°C or 68°F) generates a 6% increase in energy expenditure^{14,17}. Additionally, prolonged exposure to warm temperature (27°C or 80°F) completely inhibits BAT activity¹⁸. Thus, the whole room calorimeters are versatile tools to measure the effects of temperature modulation on energy expenditure with minimal inconvenience to study participants.

2 OBJECTIVES

2.1 Aim 1: Characterization of the energy metabolism profiles of cancer patients with and without evidence of BAT activation

In this aim we will perform detailed physiologic characterization of the energy expenditure of cancer patients with evidence of BAT activation compared with age-, sex-, and primary malignancy matched patients without evidence of BAT activation. We predict that BAT-positive patients will display a higher resting energy expenditure when compared to controls. This finding will support our hypothesis that maladaptive BAT activation may contribute to the negative energy balance of cancer-associated cachexia.

2.2 Aim 2: Assessment of environmental modulation as an effective strategy to mitigate maladaptive BAT activation in patients with malignancy.

Cancer patients with evidence of BAT activation compared with age-, sex-, and primary malignancy matched patients without evidence of BAT activation will be exposed to warm temperature and to assess the difference in energy expenditure compared to room temperature. We predicted that BAT-positive cancer subjects will display a greater decrease in energy expenditure than BAT-negative controls when exposed to warm temperature. This finding will support our hypothesis that maladaptive BAT activation may be quenched by environmental modulation, which could prove a novel, inexpensive and effective means to ameliorate or treating cancer associated cachexia.

3 STUDY DESIGN

3.1 General Description

Patients who consent to participate in this pilot study will be asked to adhere to an overnight fast prior to coming to the CRSU. After recording anthropometrics and body mass composition by Bioelectric Impedance Analysis (BIA), study volunteers dressed in light clothing (a hospital gown) will enter the whole room calorimeter. Each individual will undergo two 3-hour energy expenditure recordings in a randomized sequence per individual: 1) 3-hour baseline assessment of resting energy expenditure at 24°C (75°F, same as the room temperature at the PET/CT scan room at VCUHealth), and 2) 4-hour assessment of resting energy expenditure with temperature modulation (where the temperature in the whole room calorimeter will be increased to 27°C (80°C)²⁰).

During the screening visit, study volunteers will have their body composition measured via bioelectrical impedance analysis (BIA), and will be asked to complete the validated, 12-item, Functional Assessment of Anorexia/Cachexia Therapy (FAACT) subscale questionnaire²¹ (referred to as "Quality of Life (QoL) Questionnaire"). At each energy recording session, participants will be asked to complete a thermal comfort questionnaire before and after each session. Infrared photography of the supraclavicular region will be performed before and after each energy recording session for each participant. Urine and blood collection will occur before and after each recording session; these samples will be analyzed in this study and will also be stored for future research use.

3.2 Primary Endpoints

- 3.2.1 Difference in resting energy expenditure between BAT-positive and BAT-negative patients with cancer.
- 3.2.2 Difference in energy expenditure between room temperature and response to warm exposure (Δ energy expenditure) in BAT-positive and BAT-negative cancer patients.

The data obtained will provide a point estimate to project the metabolic effects of BAT inhibition on energy balance for larger intervention studies. We hypothesize that the BAT-positive patients with cancer will have a decrease in resting energy expenditure when they are placed in a warmer temperature (27°C) in comparison to the normal room temperature (24°C). In addition, comparing to the BAT-positive cancer patient group, the BAT-negative cancer group will have either less of a decrease or no decrease in resting energy expenditure.

3.3 Secondary Endpoints

- 3.3.1 Difference in perceived quality of life between BAT-positive and BAT-negative patients with cancer.
- 3.3.2 Difference in perceived thermal comfort before and after REE sessions between BAT-positive and BAT-negative patients with cancer.
- 3.3.3 Difference in serum values of glucose, lipids, free fatty acid and exosomes between BAT-positive and BAT-negative patients with cancer

3.3.4 Difference in urine values of creatinine, cortisol, electrolytes, catecholamines, and metanephrines between BAT-positive and BAT-negative patients with cancer.

4 PATIENT SELECTION

4.1 Inclusion Criteria

A potential subject must meet all of the following inclusion criteria to be eligible to participate in the study.

- 4.1.1 ≥ 40 years of age
- 4.1.2 Have active cancer diagnosis.
- 4.1.3 Have had a PET/CT scan within the past 36 months at time of enrollment.
- 4.1.4 Ability to understand and the willingness to sign a written informed consent document.

4.2 Exclusion Criteria

A potential subject who meets any of the following exclusion criteria is ineligible to participate in the study.

- 4.2.1 Current use of beta-blockers
- 4.2.2 Women who are pregnant or unsure of their pregnancy status
- 4.2.3 Women who are breastfeeding
- 4.2.4 Suffers from severe claustrophobia
- 4.2.5 Diagnosed with a serious psychiatric condition which could impede the judgement of the investigators, and/or the successful conduct of the recording.
- 4.2.6 In remission stage for cancer diagnosis

5 STUDY ENTRY AND WITHDRAWAL PROCEDURES

5.1 Study Entry Procedures

5.1.1 Required Pre-Registration Screening Tests and Procedures

The following 2 groups will be recruited via physician referral and flyers and the VCU Telegram (content of flyer and Telegram will be identical) sent to the MPC and MCV campuses. Self-referral will be allowed.

- | | |
|---------|--|
| Group 1 | Patients with known malignancy and incidental finding of PET/CT scans positive for BAT. |
| Group 2 | Patients with known malignancy and no evidence of BAT activity
PET/CT scans to be matched to group 1 for primary tumor and stage, sex, age (± 5 years), BMI (± 3 Kg/m ²). |

During the screening visit, the following procedures will be performed: informed consent, history and physical examination performed by a trained physician, weight and vital signs recorded by a trained physician or nurse, bio-impedance analysis, and one cachexia-specific quality of life questionnaire.

5.1.2 Registration Process

After obtaining informed consent and all initial screening visit procedures, patients will participate in two four-hour recording sessions in the whole room indirect calorimeter. The two sessions must take place within one month of each other to avoid confounding variable like environmental temperature changes and disease progression.

5.2 Study Withdrawal Procedures

5.2.1 A patient is free to withdraw from the study for any reason, at any time, without reason for doing so and without any penalty of prejudice.

5.2.2 A patient may be removed from treatment for one of the following criteria:

5.2.2.1 The study physician thinks it's necessary for the health and safety of the patient.

5.2.2.2 Principal Investigator's decision to discontinue the study.

5.2.2.3 The patient is non-compliant with the protocol

5.2.2.3 Administrative reasons that require the patient to withdrawal

5.2.3 All available data will be collected if feasible. The reason for discontinuation should be documented in the case report form (CRF).

If a patient decides to withdraw from the study, any information and specimens already collected will be handled according the following algorithm. Patients who withdrew consent before the end of their involvement will not have their data used.

Table 2: Effects of Withdrawal on Data Collected.

Timing of Withdrawal	Action
After the screening visit, before either of the two REE recordings.	Data collected will not be deleted, but no additional information regarding the patient will be obtained.
After the screening visit and REE recording at room temperature, before the REE recording at increased temperature.	Data collected will not be deleted, but no additional information regarding the patient will be obtained. Information gathered can still be used in analyses.
After the screening visit and REE recording at increased temperature, before the REE recording at room temperature.	Data collected will not be deleted, but no additional information regarding the patient will be obtained.

6 TREATMENT PLAN

6.1 Study Visit Procedures

The entire protocol consists of three visits: one screening visit (Visit 1) and two energy expenditure recording visits (Visit 2 and Visit 3). The two energy expenditure recording visits will be conducted at the same time in the day, to avoid confounders related to timing and duration of fasting. The screening visit and first energy recording visit can be performed on the same day.

Informed Consent

Prior to performing any study-related procedures, the patient must sign and date an Institutional Review Board (IRB)-approved informed consent form (ICF). The informed consent process must be thoroughly documented in the patient's record.

Medical History:

The following information will be gathered from the patient and from the review of the medical records:

- Prior history of malignancy, treatments, staging.
- Prior history of cardiovascular disease, diabetes, hypertension, dyslipidemia.
- Prior surgical history
- Medication list
- Social history (use of tobacco or alcohol)
- For females: menopausal status

Physical Examination

An abbreviated physical examination will be performed, and the following organ systems will be evaluated:

- Vital signs
- Neurological exam: focal deficit, resting tremor, reflexes
- Neck: thyroid gland size and nodularity
- Lungs: sounds
- Cardiovascular: rate, rhythm, murmur, peripheral pulses
- Abdomen: tenderness, organomegaly

Weight:

Body weight will be obtained at the screening visit.

Vitals:

Blood pressure (average of two readings, seated and at rest for 10 seconds, pulse, and temperature will be documented at the screening visit.

Resting Energy Expenditure:

Resting Energy Expenditure studies how body adjusts its metabolism to different temperatures. The order of the temperature settings will be randomized for each patient. The patient will lie on a hospital bed (if in the large room) or on a recliner for four hours per session, with limited movement for two study visits (Visit 2/Visit 3). The studies are identical except for the temperature of the room that will be adjusted between 24°C (75°F) and 27°C (80°F). The temperature will remain stable for the entire procedure. The patient will be asked not to participate in any strenuous exercise 2 days prior to the session. The patient will also be required to come dressed lightly, and fasting for at least 8 hours. Visit 2 and 3 will be scheduled at the same time in the day to avoid confounders associated with sleep cycle and duration of fasting.

Infrared Photography:

Infrared photographs from the mandible to the chest above the nipples will be taken immediately before and after each session. Infrared photography allows us to measure the temperature of the skin and the changes in blood flow and brown fat activity when exposed to different temperatures. Pictures will not expose the patient's face or breast. Infrared photography is important because it allows us to see how brown fat activity changes after each session.

Physiology Sensors and Telemetry:

Telemetry physiology sensors will be placed on the skin until the end of each session. The sensors measure the electrical activity of muscles, movements, heart rate and electrical activity, and skin temperature. The sensors are important because they allow us to determine if any other factors such as muscle movement may be contributing to measured energy expenditure during the sessions.

Blood Collection:

Approximately 12mL of blood will be collected in tubes with no additives except serum separator before and after each session. No more than 50ml of blood will be collected over the course of the two sessions. Serum will be separated by centrifugation, aliquoted in 1 mL tubes, and stored in -80°C freezers in Dr. Celi's laboratory in Sanger Hall. Samples will be analyzed for glucose, lipids, free fatty acids, exosomes, and other components for hormonal and metabolic factors related to metabolism, cancer, and cancer-associated cachexia. Samples will be stored as described above for future analyses for studies related to metabolism, cancer, and cancer-associated cachexia.

Urine Collection:

Patients will be asked to empty their bladder before the energy expenditure recording, and urine will be collected at the end of the recording to allow for a timed collection. Urine samples will be analyzed for creatinine, cortisol, electrolytes, catecholamines, and metanephrines. Urine aliquots will be stored in -80°C freezers in Dr. Celi's laboratory in Sanger Hall for future analyses for studies related to metabolism, cancer, and cancer-associated cachexia.

Bio-impedance Analysis (BIA):

BIA allows body composition (amount of fat and muscle) to be estimated. This is important because body composition may impact resting energy expenditure. The test will last approximately 5-10 minutes and will take place at the screening visit.

Quality of Life (QoL) Questionnaire:

The validated Functional Assessment of Anorexia/Cachexia Therapy (FAACT) Questionnaire will be administered. Questions will be specifically related to cachexia. The answers to the questionnaire will be used to understand the patient's current opinion on their quality of life. Questionnaire will be completed at the screening visit.

Thermal Comfort Questionnaires:

Thermal Comfort questionnaires are important in understanding if the patient felt colder or warmer during the session, as this may relate to metabolism. The questionnaire will be completed before and after each session.

7 ADVERSE EVENTS: DEFINITIONS AND REPORTING REQUIREMENTS

7.1 Definitions

7.1.1 Adverse Event (AE)

AE means any untoward medical occurrence associated with the use of a drug in humans, whether or not considered drug related.

7.1.2 Serious AE (SAE)

An AE is considered “serious” if, in the view of the investigator, it results in any of the following outcomes:

- death,
- a life-threatening AE (An AE is considered “life-threatening” if, in the view of the investigator, its occurrence places the patient or subject at immediate risk of death. It does not include an AE that, had it occurred in a more severe form, might have caused death.),
- inpatient hospitalization or prolongation of existing hospitalization,
- a persistent or significant incapacity or substantial disruption of the ability to conduct normal life functions, or
- a congenital anomaly/birth defect.

Important medical events that may not result in death, be life-threatening, or require hospitalization may be considered serious when, based upon appropriate medical judgment, they may jeopardize the patient or subject and may require medical or surgical intervention to prevent one of the outcomes listed in this definition.

7.1.3 Unanticipated Problem (UP)

Unanticipated problems include any incident, experience, or outcome that meets all of the following criteria:

- unexpected (in terms of nature, severity, frequency) given (a) the research procedures that are described in the protocol-related documents, such as the IRB-approved research protocol and informed consent document; and (b) the characteristics of the subject population being studied;
- related or possibly related to participation in the research (possibly related means there is a reasonable possibility that the incident, experience, or outcome may have been caused by the procedures involved in the research); and
- suggests that the research places subjects or others at a greater risk of harm (including physical, psychological, economic, or social harm) than was previously known or recognized.

7.1.4 AE Description and Grade

The descriptions and grading scales found in the revised Common Terminology Criteria for Adverse Events (CTCAE) version 4.0 will be utilized for AE reporting.

7.1.5 AE Expectedness

AEs can be 'Unexpected' or 'Expected'.

Expected AEs are listed in section 8.2 below.

Unexpected AEs are those AEs occurring in one or more subjects participating in the research protocol, the nature, severity, or frequency of which is not consistent with either:

- The known or foreseeable risk of AEs associated with the procedures involved in the research that are described in (a) the protocol-related document, such as the IRB-approved research protocol, any applicable investigator brochure, and the current IRB-approved informed consent document, and other relevant sources of information, such as product labeling and package inserts; or
- The expected natural progression of any underlying disease, disorder, or condition of the subject(s) experiencing the AE and the subject's predisposing risk factor profile for the AE.

7.1.6 AE Attribution

- Definite – The AE *is clearly related* to the study treatment.
- Probable – The AE *is likely related* to the study treatment.
- Possible – The AE *may be related* to the study treatment.
- Unlikely – The AE *is doubtfully related* to the study treatment.
- Unrelated – The AE *is clearly NOT related* to the study treatment.

7.2 Known AEs List

Table 3: Known AEs/Risks.

Assessment/Procedure	Possible AEs/risks
Energy expenditure recording	The only risk is anxiety or discomfort due to extended period of fasting (about 8-hours overnight fasting and 4 hours fasting during the study) and limited space.
Temperature modulation	Exposure to different environmental temperatures may be unpleasant. Some people are affected strongly by heat and may sweat a lot,

	experience low blood pressure, heavy breathing, heart palpitations, and/or dizziness.
Infrared photography	There is minimal risk associated with this procedure. The pictures will be limited to the area between the jaw and the chest above the nipples
Physiology sensors and telemetry	There is minimal risk associated with this procedure. Some individuals who are allergic or sensitive to adhesive or latex may experience skin rash (dermatitis) from the electrodes.
Blood collection	There may be pain at the site where the catheter is inserted, and some bruising and very rarely infection may occur. Some people may experience nausea or faint when the catheter is inserted or when they see blood. Bandages used to cover the catheter site may cause a skin rash (dermatitis) in some people who are allergic or sensitive to adhesive or latex
Urine collection	There is minimal risk associated with this procedure. All urine will be collected in a private location.
Bio-Impedance Analysis (BIA)	There is minimal risk associated with this procedure. Some individuals who are allergic or sensitive to adhesive or latex may experience skin rash (dermatitis) from the electrodes.
Quality of Life (QoL) questionnaires	There is minimal risk associated with this procedure. Participants may experience some emotional discomfort as they assess their perceived quality of life
Thermal comfort questionnaires	There are minimal risks or discomforts associated with this procedure.

Pregnancy: Pregnancy is not considered an AE, although a patient will be withdrawn from the study if a pregnancy occurs and the visit will be completed. The pregnancy must be immediately reported to the Principle investigator. Additional follow-up may be required.

7.3 Time Period and Grade of AE Capture

Collection of AEs will start immediately following signing of the ICF and will continue throughout the study. Illnesses present before the patient signs the informed consent form (ICF) are considered pre-existing conditions and are documented on the medical history worksheet and in the Case Report Form. Pre-existing conditions that worsen during the study are entered on the AE case report form.

Table 4 Adverse Event Grades Based on the Common Terminology Criteria for Adverse Events

Grade	Description
1	Mild: asymptomatic or mild symptoms; clinical or diagnostic observations only; intervention not indicated

2	Moderate: minimal, local, or noninvasive intervention indicated; limiting age appropriate instrumental activities of daily living (eg, preparing meals, shopping for groceries or clothes, using the telephone, and managing money)
3	Severe: severe or medically significant but not immediately life-threatening; hospitalization or prolongation of hospitalization indicated; disabling; limiting self-care activities of daily living (eg, bathing, dressing and undressing, feeding self, using the toilet, taking medications, and not bedridden)
4	Life-threatening: Life-threatening consequences; urgent intervention indicated
5	Death: Death related to AE

Source: National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events (CTCAE), version 4.03 (CTCAE 2010).

Table 5: Adverse Event Grading For Known AE/Risks Using CTCAE Guidelines

Adverse Event	Description	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
Anxiety	A disorder characterized by apprehension of danger and dread accompanied by restlessness, tension, tachycardia, and dyspnea unattached to a clearly identifiable stimulus.	Mild symptoms; intervention not indicated	Moderate symptoms; limiting instrumental ADL	Severe symptoms; limiting self care ADL; hospitalization indicated	Life-threatening consequences; urgent intervention indicated	N/A
Hyperhidrosis	A disorder characterized by excessive sweating.	Limited to one site (palms, soles, or axillae); self care interventions	Involving >1 site; patient seeks medical intervention; associated with psychosocial impact	Associated with electrolyte/hemodynamic imbalance	N/A	N/A
Palpitation	A disorder characterized by an unpleasant sensation of irregular and/or	Mild symptoms; intervention not indicated	Intervention indicated	N/A	N/A	N/A

	forceful beating of the heart.					
Pre-syncope	A disorder characterized by an episode of lightheadedness and dizziness which may precede an episode of syncope.	N/A	Present (e.g., near fainting)	N/A	N/A	N/A
Syncope	A disorder characterized by spontaneous loss of consciousness caused by insufficient blood supply to the brain.	N/A	N/A	Fainting; orthostatic collapse	N/A	N/A
Pain of Skin	A disorder characterized by a sensation of marked discomfort in the skin.	Mild pain	Moderate pain; limiting instrumental ADL	Severe pain; limiting self care ADL	N/A	N/A
Contact Dermatitis	A disorder characterized by a rash and/or itching of the skin where adhesive or latex was in contact with the skin.	Asymptomatic or mild symptoms; clinical or diagnostic observations only; intervention not indicated	Moderate; minimal, local or noninvasive intervention indicated; limiting age-appropriate instrumental ADL	N/A	N/A	N/A
Bruising	A finding of injury of the soft tissues or bone characterized by leakage of blood into surrounding tissues.	Localized or in a dependent area	Generalized	N/A	N/A	N/A
Vascular access complication		TPA administration into line with no intent for systemic	Device dislodgement, blockage, leak, or malposition; device	Pulmonary embolism, deep vein or cardiac thrombosis; intervention	Life-threatening consequences with hemodynamic or	Death

		therapy indicated	replacement indicated	indicated (e.g., anticoagulation, lysis, filter, invasive procedure)	neurologic instability	
Nausea	A disorder characterized by a queasy sensation and/or the urge to vomit.	Loss of appetite without alteration in eating habits	Oral intake decreased without significant weight loss, dehydration or malnutrition	Inadequate oral caloric or fluid intake; tube feeding, TPN, or hospitalization indicated	N/A	N/A
Vomiting	A disorder characterized by the reflexive act of ejecting the contents of the stomach through the mouth.	Intervention not indicated	Outpatient IV hydration; medical intervention indicated	Tube feeding, TPN, or hospitalization indicated	Life-threatening consequences	Death

7.4 Procedures for Recording AEs, SAEs, and Ups

All AEs will be documented on the AE case report form and in the patient's medical record. The following attributes must be assigned: (1) description, (2) dates of onset and resolution, (3) severity, (4) "serious" criteria if applicable, and (5) action taken. The investigator will actively solicit this information and assess the AEs in terms of severity and relationship to the study. The Investigator will treat the patient as medically required until the AE either resolves or becomes medically stable. The treatment may extend beyond the duration of the study. The investigator will record treatment and medications required for treatment on the appropriate CRF(s).

In the event that a patient is withdrawn from the study because of an AE, the event must be recorded on the Termination CRF as the reason for discontinuation.

7.5 Routine Reporting Procedures for AEs

Each event will be reported to the Principle Investigator once becoming aware of the occurrence. All AEs and SAEs will be followed until resolution, until the condition stabilizes, until the event is otherwise explained, whichever occurs first. All AEs and SAEs documented at a previous visit/contact and designated as ongoing, will be reviewed at subsequent visits/contacts, where the designation may remain ongoing. The investigator will ensure that the follow-up includes any supplemental investigation as may be indicated to elucidate the nature and/or causality of the SAE. This may include additional laboratory test or investigations, histopathological examinations, or consultation with other

health care professionals. SAEs that are ongoing at the time of the subjects final study visit/contact will be documented as ongoing.

7.6 Expedited Reporting Procedures for SAEs, UPs, and DLTs

Expedited Reporting Requirements (Events, Report Recipients, and Time Frames)	
SAEs	UPs
Principal Investigator ¹	Principal Investigator ¹
	DSMB ¹
	IRB ²
¹ Report event within 5 business days of becoming aware of the occurrence. ² Each UP must be reported to the VCU IRB within 5 business days of becoming aware of the occurrence. The report must be prepared using the "VCU IRB PROMPT REPORTING FORM," found on the VCU IRB Forms Page.	
Principal Investigator Francesco S. Celi, MD, MHSc 804-828-8389 Francesco.celi@vcuhealth.org	Massey Cancer Center DSMB FAX: 804-828-5406 E-mail: masseydsmb@vcu.edu

8 STUDY CALENDAR

This is a more detailed version of **Table 1: Schedule of Events** from the section: **Schema**.

Assessment /Procedure	Screening Visit	Visit 2	Visit 3
Informed Consent	X		
Confirm Inclusion/Exclusion	X		
Medical History ^a	X		
Physical Examination	X		
Weight	X		
Vital Signs ^b	X		
Bio-Impedance Analysis ^c	X		
QOL questionnaires	X		
Resting Energy Expenditure ^d		X	X
Infrared Photography ^e		X	X
Physiology Sensors and Telemetry ^f		X	X
Blood Collection ^g		X	X
Urine Collection ^h		X	X
Thermal Comfort Questionnaires		X	X

- a. Medical history will include mental health and/or psychiatric history
- b. Blood pressure, pulse, and temperature
- c. Measures amount of fluid, fat, and muscle content using a very low electrical current.
- d. Patients are allowed to watch TV, read a book, use phone or other small electronics, but movement should be limited. Must be fasting for 8 hours, and must not have participated in any strenuous exercise two days prior.
- e. Must be taking immediately before and after each session. Pictures are taking from mandible to the chest above the nipples.
- f. Will remain for the entire study session. Sensors will be connected to transmitters to monitor electrical activity of muscles, movements, heart rate and electrical activity, and skin temperature.
- g. Blood sample for analysis of glucose, lipids, free-fatty acids, exosomes, and other components for hormonal and metabolic factors related to metabolism, cancer, and cancer-associated cachexia.
- h. Urine sample for analysis of creatinine, cortisol, electrolytes, catecholamines, metanephrines, and other hormones.

9 STATISTICAL CONSIDERATIONS

9.1 Study Design and Analysis

The primary endpoint of this study is to determine whether resting energy expenditure decreases in BAT-positive cancer patients when the room temperature increases, as a reflection of the quenching of mal-adaptive activation of BAT. The subjects will rest in whole-room calorimeters for 4 hours each day for two days (one day with room temperature set at 24°C, the other 27°C), with their minute-by-minute resting energy expenditure recorded while fasting. Since resting energy expenditure during fasting period has little variation, we propose to average the resting energy expenditure on each day, and conduct a one-sample, one-sided t-test to compare the average resting energy expenditure on both days. To determine whether there is a difference between BAT-positive group and the BAT-negative group undergoing the same experiment procedure. For this endpoint, we will conduct a two-sample, two-sided t-test to compare the change in average resting energy expenditure of the BAT-positive group and the BAT-negative group.

9.2 Sample Size/Accrual Rates

Statistical Power Analysis. From previous study in healthy subjects, cold exposure caused energy expenditure increase due to BAT activation is 5%~7%. If the reverse effect of warm exposure on energy expenditure due to BAT quenching holds true, we could assume similarly, percentage drop in energy expenditure in BAT positive group might also be 5%~7%, whereas this percentage drop in BAT negative group is 0%. Assuming the natural variation of energy expenditure in BAT negative group is 5%, the standardized effect size is 1 ~ 1.4. Considering that comparing the difference between groups requires larger sample size, we use a two-sample, one-sided t-test to calculate the sample size. This means to reach a statistical power of 80%, a sample size of 14 subjects per group is required to reject the null hypothesis assuming a type I error of 0.05. Considering the potential dropout in this two-day study, we assume a retention rate of 80%. This means a conservative sample size of 18 subjects per group (BAT-positive and BAT-negative) is required.

36 subjects over a period of two years

- | | |
|---------|--|
| Group 1 | 18 patients with known malignancy and incidental finding of PET/CT scans positive for BAT. |
| Group 2 | 18 patients with known malignancy and no evidence of BAT activity PET/CT scans. Patients will be matched to group 1 for primary tumor and stage, sex, age (± 5 years), BMI (± 3 kg/m ²). |

We expect to recruit about 2 patients per month into the study.

Withdrawals will be handled as described in Section 5.2.

9.3 Stratification Factors

No stratification is planned in this study.

9.4 Analysis of Secondary Endpoints

The secondary endpoints (see Section 3.4) aim to compare the difference between the BAT-positive and BAT-negative patients with cancer regarding their quality of life (Section 3.4.1), thermal comfort during the experiment (Section 3.4.2), biomarkers in the blood serum (Section 3.4.3), and biomarkers in the urine samples (Section 3.4.3). Specially, to compare the quality of life (assessed by a questionnaire containing only ordinal data) in the two groups, we will conduct Mann-Whitney U-test on each questionnaire item as well as the total quality of life score. To compare the thermal comfort change (assessed by a questionnaire with a single question) before and after the REE sessions in the two groups, we will conduct Mann-Whitney U-test on the thermal comfort change. To compare the difference in the blood samples and urine samples, we will use two-sample, two-sided t-test for each biomarker.

10 DATA AND SAFETY MONITORING PLAN (DSMP)

10.1 Study Team

The DSMP for this study will consist of a single element, a study team: The study team minimally consists of the principal investigator, the clinical research coordinator, the research assistant, and the study biostatistician. The principle investigator (PI) will instruct the study personnel during a meeting prior to the initiation of the study and every 6 months thereafter. The meeting will describe in detail the purpose of the study, rationale, hypothesis, and research conduct. The study personnel will report any unanticipated adverse events, or any other problem with the study to the PI. The PI is directly responsible for the conduct and monitoring of study procedures. The PI will supervise all study procedures, including the informed consent process, blood sample collection and analysis. The PI will be available to all study personnel to answer questions.

11 REGULATORY COMPLIANCE AND ETHICS

11.1 Ethical Standard

This study will be conducted in conformance with the principles set forth in *The Belmont Report: Ethical Principles and Guidelines for the Protection of Human Subjects of Research* (US National Commission for the Protection of Human Subjects of Biomedical and Behavioral Research, April 18, 1979).

11.2 Regulatory Compliance

This study will be conducted in compliance with:

- The protocol
- Federal regulations, as applicable, including: 21 CFR 50 (Protection of Human Subjects/Informed Consent); 21 CFR 56 (Institutional Review Boards), and 45 CFR 46 Subparts A (Common Rule), B (Pregnant Women, Human Fetuses and Neonates), C (Prisoners), and D (Children)

11.3 Institutional Review Board

Each participating institution must provide for the review and approval of this protocol and the associated informed consent documents and recruitment material by an appropriate IRB registered with the Office for Human Research Protections (OHRP). Any amendments to the protocol or consent materials must also be approved. In the United States and in other countries, only institutions holding a current US Federal wide Assurance issued by OHRP may participate.

11.4 Informed Consent Process

Informed consent is an ongoing process that is initiated prior to the individual's agreeing to participate in the study and continues throughout the individual's study participation. Extensive discussion of risks and possible benefits of this therapy will be provided to the subjects and their families. Consent forms describing in detail the study interventions/products, study procedures, and risks are given to the subject and written documentation of informed consent is required prior to starting intervention/administering study product. Consent forms will be IRB-approved and the subject will be asked to read and review the document. Upon reviewing the document, the investigator will explain the research study to the subject and answer any questions that may arise. The subject will sign the informed consent document prior to any procedures being done specifically for the study. The subjects should have the opportunity to discuss the study with their surrogates or think about it prior to agreeing to participate. The subjects may withdraw consent at any time throughout the course of the trial. A copy of the informed consent document will be given to the subjects for their records. The rights and welfare of the subjects will be protected by emphasizing to them that the quality of their medical care will not be adversely affected if they decline to participate in this study.

11.5 Subject Confidentiality and Access to Source Documents/Data

Subject confidentiality is strictly held in trust by the participating investigators and their staff. This confidentiality includes the clinical information relating to participating subjects, as well as any genetic or biological testing.

The study protocol, documentation, data, and all other information generated will be held in strict confidence. No information concerning the study or the data will be released to any unauthorized third party without prior written approval of the principal investigator.

The principal investigator will allow access to all source data and documents for the purposes of monitoring, audits, IRB review, and regulatory inspections.

The study monitor or other authorized representatives of the principal investigator may inspect all documents and records required to be maintained by the investigator, including but not limited to, medical records (office, clinic, or hospital) and pharmacy records for the subjects in this study. The clinical study site will permit access to such records.

12 DATA HANDLING AND RECORD KEEPING

12.1 Data Management Responsibilities

The principal investigator is responsible for: (i) the overall conduct of the investigation; (ii) ongoing review of trial data including all safety reports; and (iii) apprising participating sites of any UPs.

Any laboratory conducting correlative studies must maintain the laboratory records and documentation (laboratory notebooks, laboratory protocols, print-outs, recordings, photographs, etc.).

12.2 Source Documents

Paper based source documents will be kept in secure location and only accessed by authorized study personnel. Electronic records will be made available only to those personnel in the study through the use of access controls and encryption. Identifiers will be removed from study-related data (data is coded with a key and stored in a separate and secure location).

12.3 Case Report Forms

Patient information will be collected and documented in paper based case report forms. All hand written entries on the case report forms (CRFs) should be made legibly in black ink. Errors, when made, should be corrected by drawing a single line through the incorrect entry (do not erase, white-out, or tape over errors) and then entering the correct data above the original entry. Entry corrections should be initialed and dated. Explain missing data with "ND" for "not determined" and "NA" used for "not applicable."

12.4 Study Record Retention

As applicable, study records will be maintained a minimum of 5 years beyond: (i) the publication of any abstract or manuscript reporting the results of the protocol; (2) the submission of any sponsored research final report; or (iii) submission of a final report to clinicaltrials.gov. Those patients who consent to be a part of the data registry will have all personal identifiers kept indefinitely. Information in the databases will only be accessible to individuals working on the study or VCU/VCUHS officials who have access for specific research-related tasks.

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Appendix C

Human Subjects Research Quality of Life Questionnaire

FAACT (Version 4)

Below is a list of statements that other people with your illness have said are important. **Please circle or mark one number per line to indicate your response as it applies to the past 7 days.**

		Not at all	A little bit	Some- what	Quite a bit	Very much
<u>PHYSICAL WELL-BEING</u>						
GP1	I have a lack of energy	0	1	2	3	4
GP2	I have nausea	0	1	2	3	4
GP3	Because of my physical condition, I have trouble meeting the needs of my family	0	1	2	3	4
GP4	I have pain	0	1	2	3	4
GP5	I am bothered by side effects of treatment	0	1	2	3	4
GP6	I feel ill	0	1	2	3	4
GP7	I am forced to spend time in bed	0	1	2	3	4
<u>SOCIAL/FAMILY WELL-BEING</u>						
GS1	I feel close to my friends	0	1	2	3	4
GS2	I get emotional support from my family	0	1	2	3	4
GS3	I get support from my friends	0	1	2	3	4
GS4	My family has accepted my illness	0	1	2	3	4
GS5	I am satisfied with family communication about my illness	0	1	2	3	4
GS6	I feel close to my partner (or the person who is my main support)	0	1	2	3	4
Q1	<i>Regardless of your current level of sexual activity, please answer the following question. If you prefer not to answer it, please mark this box <input type="checkbox"/> and go to the next section.</i>					
GS7	I am satisfied with my sex life	0	1	2	3	4

FAACT (Version 4)

Please circle or mark one number per line to indicate your response as it applies to the past 7 days.

<u>EMOTIONAL WELL-BEING</u>		Not at all	A little bit	Some- what	Quite a bit	Very much
GE1	I feel sad	0	1	2	3	4
GE2	I am satisfied with how I am coping with my illness.....	0	1	2	3	4
GE3	I am losing hope in the fight against my illness.....	0	1	2	3	4
GE4	I feel nervous	0	1	2	3	4
GE5	I worry about dying	0	1	2	3	4
GE6	I worry that my condition will get worse	0	1	2	3	4

<u>FUNCTIONAL WELL-BEING</u>		Not at all	A little bit	Some- what	Quite a bit	Very much
GF1	I am able to work (include work at home).....	0	1	2	3	4
GF2	My work (include work at home) is fulfilling.....	0	1	2	3	4
GF3	I am able to enjoy life.....	0	1	2	3	4
GF4	I have accepted my illness.....	0	1	2	3	4
GF5	I am sleeping well	0	1	2	3	4
GF6	I am enjoying the things I usually do for fun	0	1	2	3	4
GF7	I am content with the quality of my life right now.....	0	1	2	3	4

FAACT (Version 4)

Please circle or mark one number per line to indicate your response as it applies to the past 7 days.

<u>ADDITIONAL CONCERNS</u>		Not at all	A little bit	Some- what	Quite a bit	Very much
C6	I have a good appetite.....	0	1	2	3	4
ACT1	The amount I eat is sufficient to meet my needs.....	0	1	2	3	4
ACT2	I am worried about my weight.....	0	1	2	3	4
ACT3	Most food tastes unpleasant to me.....	0	1	2	3	4
ACT4	I am concerned about how thin I look.....	0	1	2	3	4
ACT6	My interest in food drops as soon as I try to eat.....	0	1	2	3	4
ACT7	I have difficulty eating rich or "heavy" foods.....	0	1	2	3	4
ACT9	My family or friends are pressuring me to eat	0	1	2	3	4
O2	I have been vomiting	0	1	2	3	4
ACT1 0	When I eat, I seem to get full quickly	0	1	2	3	4
ACT1 1	I have pain in my stomach area	0	1	2	3	4
ACT1 3	My general health is improving.....	0	1	2	3	4

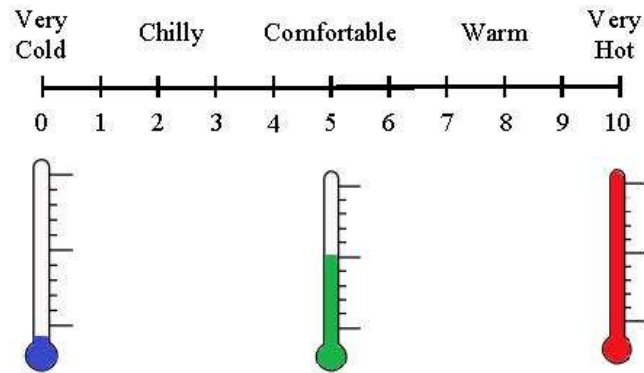
Appendix D

Human Subjects Research Thermal Comfort Questionnaire

Thermal Comfort Questionnaire

Please circle or mark ONE number to indicate your response as it applies to you at this moment.

How comfortable is your body temperature?



Appendix E

Human Subjects Research Informed Consent Document

RESEARCH PARTICIPANT INFORMATION AND CONSENT FORM

STUDY TITLE: Maladaptive adipose tissue activity in cancer

VCU IRB PROTOCOL NUMBER: HM20009089

INVESTIGATORS: Francesco S. Celi, MD, MHSc
Janina Vaitkus Pearce, MD-PhD Student
Egidio Del Fabbro, MD
Angeliki Stamatouli, MD
Martin Charron, MD
Tamas Gal, PhD, MS
Shanshan Chen, PhD

SPONSOR: Virginia Commonwealth University Massey Cancer Center Pilot Project

What are some general things you should know about research studies?

In this consent form, "you" always refers to the research participant. You are being asked to take part in a research study. To join this study is voluntary. You may refuse to join, or you may withdraw your consent to be in the study, for any reason, without penalty.

Research studies are designed to obtain new knowledge. This new information may help people in the future. You may not receive any direct benefit from being in the research study. There also may be risk to being in research studies. Deciding not to be in the study or leaving the study before it is done will not affect your relationship with the researcher, your health care provider, or Virginia Commonwealth University. If you are a patient with an illness, you do not have to be in the research study in order to receive health care.

Details about this study are discussed below. It is important that you understand this information so that you can make an informed choice about being in this research study.

Please ask the study doctor or the study staff to explain any information in this consent document that is not clear to you at any time. You may take home an unsigned copy of this consent form to think about or discuss with family or friends before making your decision.

Description of the study

Energy expenditure is the amount of energy a person uses to carry out a physical function, such as breathing or exercising. The measuring of energy expenditure is important in understanding how the body maintains energy balance, which is the relationship between how much food (energy) a person eats and how energy a person uses during different activities (for example, sleeping, resting, or exercising).

This information is important because it will help researchers figure out how to help prevent and treat diseases such as cancer-associated cachexia. Virginia Commonwealth University has indirect calorimeter rooms that measure the oxygen used and the carbon dioxide produced by the person in the room as they breathe. This information is used to calculate the energy expenditure and type of fuel (sugar, fat, or protein) used.

Whole Room Indirect Calorimeters

Whole room indirect calorimeters are rooms that can measure the calories, or energy you burn; this measurement is called "energy expenditure". To conduct the measurement, we analyze continuously the oxygen and carbon dioxide content of the air that you breathe out while you are resting in a special room called "indirect calorimeter". This type of rooms are no different from a regular living room, except they are sealed to maintain and are supplied with constant fresh medical grade air to exchange expired air. In our study, we will use the two whole room indirect calorimeters located in the Clinical Research Service Unit (CRSU) on the 8th floor of the North Hospital, on the MCV campus of Virginia Commonwealth University. The first indirect calorimeter room (called the large room in this form) is about 10' x 10'. This room has a bed, desk, chair or recliner, treadmill or stationary bicycle, and toilet and sink. The smaller indirect calorimeter room (called the flex room) is about 8' x 4', equipped with a recliner, or a mattress with bed boxes. Both rooms are equipped with nurse call buttons and a wireless intercom system to allow communication with nurses, physicians, and study staff. An environmental control unit is installed outside the rooms, and the study staff will adjust the temperature according to the study procedure.

Both rooms have no locks, so you may leave at any time. The major difference between these rooms is that the flex room is used for shorter studies, and the large room is used for both short and long studies up to three days. For this research study, we will take measurements in either the large or flex room.

What is the purpose of the study?

The purpose of this research is to study brown adipose tissue, a type of fat that increases metabolism (burns energy) during exposure to cold, and how it may contribute to the weight loss observed in cancer. Many patients affected by cancer lose weight, despite normal or increased nutrition. It may progress to the condition called "cancer-associated cachexia." Cancer-associated cachexia is defined by an ongoing loss of skeletal muscle mass (with or without the loss of fat mass, and may negatively affect quality of life and the ability to undergo cancer treatments. Metabolism is the breakdown of food by the body into the energy that your body needs.

You are being asked to participate in this study because you have a known malignancy and incidental finding of PET/CT scans positive for brown adipose tissue, have a known malignancy and no evidence of brown adipose tissue activity PET/CT scans, have no known malignancy, are 40 years or older, not pregnant, not breastfeeding, not taking beta-blockers (a class of drugs used to treat hypertension and heart disease), and do not have any serious psychiatric conditions. If you have claustrophobia (fear of being in enclosed spaces), or other psychiatric conditions that the investigators think would interfere with the study procedures, you will not be able to participate in this study.

How Many people will take part in this study?

Approximately thirty six participants will take part in this study.

What will happen if you take part in this study?

If you decide to be in this research study, you will be asked to sign this consent form after you have had all your questions answered. We will clearly explain all the procedures that we are doing in this study.

Screening visit

At your first study visit (called the screening visit), we will ask you about your medical history (including your mental health and/or psychiatric history). A trained physician will perform a physical exam, and a trained physician or nurse will obtain your weight and vital signs (blood pressure, pulse, and temperature).

Bio-electrical impedance analysis (BIA) will be performed and you will be asked to fill out Quality of Life (QoL) questionnaires at the screening visit after informed consent has been obtained.

This visit will last approximately 30 to 45 minutes.

Study visits (V₁ and V₂)

If eligible after the Screening visit, a study coordinator will contact you to schedule your two study visits which are conducted in the whole room calorimeters. The first visit may be take place on the same day as the screening visit. The two visits are identical, will last approximately four and a half hours, and must be completed within one month. The study dates are relatively flexible and we will try to arrange the date and time of the study procedures to coordinate with other medical visits you may have at VCU Health. Trained personnel will perform all procedures. A physician will be present during the initial visit and will also be available for the duration of all study procedures for assistance. Physicians, nurses, or dedicated personnel will perform the blood draws

Study procedures**Resting Energy Expenditure**

We will ask you to lie on a hospital bed (if in the large room) or on a mattress (if in the flex room) for about four hours per session. During this period, you can watch TV, read a book, use your phone or other small electronics, but you should not move very much.

You will perform two of these studies which are identical except for the temperature of the room that will be adjusted between 23°C (73.4°F) and 27°C (80°F). The temperature will remain stable for the entire procedure. We will ask you to dress lightly (hospital scrubs or shorts and a light shirt), to fast for about 8 hours before your session, and to not participate in any strenuous exercise for the 2 days before you come for each session.

This studies how your body adjusts its metabolism to different temperatures. You will not know the sequence of the temperatures in the studies. This is called "randomization."

Infrared Photography

We will take infrared pictures from your jaw to your chest above the nipples. We will take the pictures immediately before and after each session.

Infrared photographs allows us to measure the temperature of your skin and the changes in blood flow and brown fat activity when you are in different temperatures. The pictures will not show your face or your breasts.

This is important to the study because it will allow us to see if your brown fat activity changes after each session. Brown fat activity is involved in your energy utilization.

Physiology Sensors and Telemetry

We will ask to place telemetry physiology sensors on your skin with adhesive tape for you to keep on during the session.

These sensors measure the electrical activity of your muscles, your movements, your heart rate and electrical activity, and your skin temperature. We will connect the sensors to transmitters (telemetry) so we can monitor them while you are in the rooms.

This is important to the study because these will tell us if there are other factors such as muscle movement that might contribute to your measured energy expenditure during each session.

Blood Collection

We will insert an intravenous flexible catheter (a tube that is about the same size and length as needles used for blood drawing) in a vein to collect blood samples at specified times while you are in the rooms.

If you have an IV port and are willing to use it for blood draw, we will ask our nursing personnel to draw blood from it rather than using your arm.

We will collect about 12mL (less than one tablespoon) of blood before and after each session, which equals about 24mL at each of the two sessions. We will collect no more than 50mL of blood over the course of the two sessions.

We will store and analyze your blood for glucose (blood sugar), lipids (fats), free fatty acids, exosomes, and other components for hormonal and metabolic factors related to metabolism, cancer, and cancer-associated cachexia. This is important because it will help us understand what factors are contributing to your measured energy expenditure.

Urine Collection

We will ask you to empty your bladder before the beginning of the procedure and to collect any urine you produce during the stay. Additionally, we will ask you to void and collect the last sample at the end of the energy expenditure recording.

The samples will be analyzed for creatinine, cortisol, electrolytes, catecholamines, metanephrines, and other hormones and to see how well your organs work. This is important for this study because it will help us understand what factors are contributing to your measured energy expenditure.

The urine will be stored for future analyses for studies related to metabolism, cancer, and cancer-associated cachexia.

Bio-impedance Analysis (BIA)

You will lie flat on a bed while four electrodes (two on the feet, two on the hands) are applied. A very low-intensity electrical current (so low that you cannot feel it) is run through your body to measure amount of water in your body.

This will allow us to estimate the amount of fat and muscle content in your body. This is important for this study because the amount of fat and muscle content in your body may impact your resting energy expenditure.

The test will last approximately 5-10 minutes and will take place at the screening visit after informed consent has been obtained.

Quality of Life (QoL) Questionnaires

We will ask you to complete two Quality of Life Questionnaires specifically related to cachexia (wasting syndrome), on paper.

The answers to these questionnaires will be used to understand your current opinion on your quality of life. These are important for this study because it will help us see if there is a link between your measured energy expenditure and your current opinion of your quality of life.

These should take no more than 15 minutes to complete and will be completed at the screening visit after informed consent has been obtained.

Thermal Comfort Questionnaires

We will ask you to indicate your thermal comfort (how comfortable you feel regarding your current temperature) before and after the completion of each session.

The answers to this questionnaire are important to understand if you felt colder or warmer during the session, as this may relate to your metabolism.

What are the possible risks or discomforts involved from being in the study?

Energy expenditure recording: The only risk is anxiety or discomfort due to extended period of fasting (about 8-hours overnight fasting and 4 hours fasting during the study) and limited space. Study staff, experienced nurses, and hospital physicians will be available to help if you become anxious. If at any time you feel too anxious or too uncomfortable, you can simply walk out of the room, as there are no locks on the doors.

Temperature modulation: Exposure to different environmental temperatures may be unpleasant. Some people are affected strongly by heat and may sweat a lot. Some may experience low blood pressure, heavy breathing, heart palpitations, and dizziness. If you feel that the temperature is too unpleasant, you can open the door and exit the room.

Infrared photography: There is minimal risk associated with this procedure. The pictures will be limited to the area between your jaw and the chest above the nipples.

Physiology sensors and telemetry: There is minimal risk associated with this procedure. Some individuals are allergic or sensitive to adhesive or latex and may experience skin rash (dermatitis) from the electrodes.

Blood collection: There may be pain at the site where the catheter is inserted. Bruising and very rarely infection may occur. Some people may experience nausea or faint when the catheter is inserted or when they see blood. Please inform the study personnel if you have experienced any of these reactions before. Bandages used to cover the catheter site may cause a skin rash (dermatitis) in some people who are allergic or sensitive to adhesive or latex.

Urine collection: There is minimal risk associated with this procedure. All urine will be collected in a private location.

Bio-Impedance Analysis (BIA): There is minimal risk associated with this procedure. Some individuals who are allergic or sensitive to adhesive or latex may experience skin rash (dermatitis) from the electrodes.

Quality of Life (QoL) questionnaires: There is minimal risk associated with this procedure. You may experience some emotional discomfort as you assess your perceived quality of life.

Thermal comfort questionnaires: There are minimal risks or discomforts associated with this procedure.

What if we learn about new findings or information during the study?

You will be informed of any significant new findings discovered during the research study that may change your willingness to continue in the study. If any of the measurements that we obtain are not normal (for example, high blood sugar or high blood pressure), you will be informed about them; and with your permission, and your primary care physician will be notified as well.

What will happen if you are injured by this research?

All Research involves a chance that something bad might happen to you. This may include the risk of personal injury. In spite of all safety measures, you might develop a reaction or injury from being in this study. If such problems occur, the researchers will help you get care, but any costs for the medical care will be billed to you and/or your insurance company. The study is of minimal risk, therefore it is not expected that there will be any harm to you that will require compensation. You do not give up any legal rights by signing this form.

USE AND DISCLOSURE OF PROTECTED HEALTH INFORMATION

Authority to Request Protected Health Information

The following people and/or groups may request my Protected Health Information:

- Principal Investigator and Research Staff
- Government/Health Agencies
- Others as Required by Law
- Study Sponsor
- Institutional Review Boards

Authority to Release Protected Health Information

The VCU Health System (VCUHS) may release the information identified in this authorization from my medical records and provide this information to:

- Health Care Providers at the VCUHS
- Study Sponsor
- Others as Required by Law
- Principal Investigator and Research Staff
- Institutional Review Boards
- Government/Health Agencies

Once your health information has been disclosed to anyone outside of this study, the information may no longer be protected under this authorization.

Type of Information that may be released

The following types of information may be used for the conduct of this research:

- | | | |
|--|--|--|
| <input checked="" type="checkbox"/> Complete health record | <input checked="" type="checkbox"/> Diagnosis & treatment codes | <input type="checkbox"/> Discharge summary |
| <input checked="" type="checkbox"/> History & physical exam | <input type="checkbox"/> Consultation reports | <input checked="" type="checkbox"/> Progress notes |
| <input checked="" type="checkbox"/> Laboratory test results | <input type="checkbox"/> X-ray reports | <input type="checkbox"/> X-ray films / images |
| <input checked="" type="checkbox"/> Photographs, videotapes | <input type="checkbox"/> Complete billing record | <input type="checkbox"/> Itemized bill |
| <input type="checkbox"/> Information about drug or alcohol abuse | <input type="checkbox"/> Information about Hepatitis B or C tests | |
| <input type="checkbox"/> Information about psychiatric care | <input type="checkbox"/> Information about sexually transmitted diseases | |
| <input checked="" type="checkbox"/> Other (specify): PET/CT reports and images | | |

Expiration of This Authorization

- ☐ This authorization will expire when the research study is closed, or there is no need to review, analyze and consider the data generated by the research project, whichever is later.
- ☒ This research study involves the use of a Data or Tissue Repository (bank) and will never expire.
- ☐ Other (specify):

What if you want to stop the use of your protected health information?

You may change your mind and revoke (take back) the right to use your protected health information at any time. Even if you revoke this authorization, the researchers may still use or disclose health information they have already collected about you for this study. If you revoke this authorization you may no longer be allowed to participate in the research study. To revoke this authorization, you must write to the Principle Investigator.

What are the possible benefits from being in this study?

This is not a treatment study, and you are not expected to receive any direct medical benefits from your participation in the study. You will be provided with your personalized resting energy expenditure point estimates at both temperatures after data analysis is complete. The information from this research study may lead to a better treatment in the future for people who have cancer or other metabolic disease.

Will it cost you anything to be in this study?

There are no charges to you for any procedures in this study. However, if you require additional medical care for research-related injuries, you or your insurance company are responsible for the related costs.

Will you receive anything for being in this study?

If you choose to enroll in this study, you will receive \$10 after completion of the screening visit, \$30 after completion of the first session, and \$60 after completion of the second session. After each study visit you will also receive a \$5 food voucher to use at our cafeteria.

Total payments within one calendar year that exceed \$600 will require the University to annually report these payments to the IRS and you. This may require you to claim the compensation you receive for participation in this study as taxable income. VCU is required by federal law to collect your social security number. Your social security number will be kept confidential and will only be used to process payment.

What other options are available if I do not take part in this study?

Since this study is not intended to provide any medical treatment or other health-related benefit, your option would be not to participate.

How will information about you be protected?

Data is being collected only for research purposes.

Potentially identifiable information about you will consist of anthropometric measures (weight, height, blood pressure, heart rate), laboratory data, and physiologic tests (for example, energy expenditure or skin temperature).

Your data will be identified by ID numbers. Access to research data will be limited to study personnel. All potential personal identifiers such as names and medical record numbers will be stored separately from the rest of the data in a locked research area and/or password-protected files. De-identified information will be stored indefinitely. All personal identifiers will either be kept indefinitely (if you consent to be part of our data registry; see next section), or destroyed five years after data publication (if you do not consent to be part of our data registry; see next section).

VCU and the VCU Health System have established secure databases to help with monitoring and oversight of clinical research. Your information may be maintained in these databases will only be accessible to individuals working on this study or VCU/VCUHS officials who have access for specific research-related tasks. Identifiable information in these databases are not released outside of VCU/VCUHS unless stated in this consent or required by law. Personal information about you might be shared with or copied by authorized officials of the Federal Food and Drug Administration or the Department of Health and Human Services.

Although results of this research may be presented at meetings or in publications, identifiable personal information pertaining to participants will not be disclosed.

You may visit the NCI website at <http://cancer.gov/> for more information about studies or general information about cancer. You may also call the NCI Cancer Information Service to get the same information at: 1-800-4-CANCER (1-800-422-6237).

Optional genetic testing and data registries

A data registry collects and stores patient medical information and other related information for use in medical research. The purpose of this registry is to collect and store medical and other information from individuals to improve knowledge of diseases such as cancer. If you join this registry, you will be asked to provide information about your health.

As mentioned above, you will not be told the genetic testing results. Results and consent documents will not be placed in your medical record. Information from this study, genetic testing, and the data registry may be shared with other researchers, but to make sure your privacy is protected, we will remove your name, address, and other potential identifiable information before sharing it with other researchers. This information is called 'de-identified' because it has all personal identifiers removed.

Only researchers approved by the Principal Investigator will be allowed to have access to the de-identified registry. Each access to the registry is recorded.

My blood samples may be used for genetic testing. I am aware that the results of the genetic testing will not be provided to me.

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Approved by the VCU IRB on 1/22/2019

My blood samples may be stored and used for future research about cancer and other metabolic diseases.

YES _____
Initials

NO _____
initials

My blood samples may be stored and used for future research about other medical problems.

YES _____
Initials

NO _____
initials

My urine samples may be stored and used for future research about cancer and other metabolic diseases.

YES _____
Initials

NO _____
initials

My urine samples may be stored and used for future research about other medical problems.

YES _____
Initials

NO _____
initials

What if you want to withdraw genetic testing consent and registry?

If you decide to withdraw the consent to the use of your genetic information and to the registry, we will destroy your stored genetic material and samples in the registry and remove all the data from ongoing studies. Please note that once we have submitted publications including data derived from your genetic information, we will not be able to remove your data from that; however, we will remove your data from any additional study. You should send a request in writing to the Principal Investigator asking to have your genetic information removed from the dataset.

What if you want to stop before your part in the study is complete?

Your participation in this study is voluntary. You may decide to not participate in this study. Your decision to not take part will result in no penalty or loss of benefits to which you are otherwise entitled. If you do participate, you may freely withdraw from the study at any time. Your decision to withdraw will result in no penalty or loss of benefits to which you are otherwise entitled. If you are a VCU/VCUHS student or employee, the participation or the decision to not participate will not affect your grades or career progression.

If you decide to stop being in the study, we ask that you allow ongoing use of the already collected specimens and information. If you choose to withdraw your specimens and data from the study, we ask that you give written instructions to destroy specimens stored in the study.

Your participation in this study may be stopped at any time by the study physicians or the sponsor without your consent. Reasons for this could include but are not limited to:

- The study physician thinks it necessary for your health or safety;
- You have not followed the study instructions;
- The sponsor has stopped the study; or
- Administrative reasons require your withdrawal.

Certificate of Confidentiality

This research will apply for a Certificate of Confidentiality from the National Institutes of Health. The researchers with this Certificate may not disclose or use information, documents, or biospecimens that may identify you in any federal, state, or local civil, criminal, administrative, legislative, or other action, suit, or proceeding, or be used as evidence, for example, if there is a court subpoena, unless you have consented for this use. Information, documents, or biospecimens protected by this Certificate cannot be disclosed to anyone else who is not connected with the research except, if there is a federal, state, or local law that requires disclosure (such as to report child abuse or communicable diseases but not for federal, state, or local civil, criminal, administrative, legislative, or other proceedings, see below); if you have consented to the disclosure, including for your medical treatment; or if it is used for other scientific research, as allowed by federal regulations protecting research subjects.

The Certificate cannot be used to refuse a request for information from personnel of the United States federal or state government agency sponsoring the project that is needed for auditing or program evaluation by Massey Cancer Center which is funding this project or for information that must be disclosed in order to meet the requirements of the federal Food and Drug Administration (FDA). You should understand that a Certificate of Confidentiality does not prevent you from voluntarily releasing information about yourself or your involvement in this research. If you want your research information released to an insurer, medical care provider, or any other person not connected with the research, you must provide consent to allow the researchers to release it.

What if you have questions about this study?

If you have any questions, complaints, or concerns about your participation in this research, please contact:

Francesco S. Celi, MD, MHSc.
Virginia Commonwealth University
Sanger Hall 7th Floor, Room 7-007
1101 East Marshall Street
Richmond, VA 23298
Phone: (804) 828-9696; (804) 628-1631
Fax: (804) 828-8389

What if you have questions about your rights as a research participant?

Call this number to ask general questions, to obtain information or offer input, and to express concerns or complaints about research. You may also call this number if you cannot reach the research team for this study, or if you wish to talk to someone else. General information about

participation in research studies can be found at
<http://www.research.vcu.edu/irb/volunteers.html>

Office of Research and Human Protection
Virginia Commonwealth University
800 East Leigh Street, Suite 3000
Box 980568
Richmond VA 23298
Phone: (804) 827-2157

Do not sign this consent form unless you have had a chance to ask questions and have received satisfactory answers to all your questions.

CONSENT

I have been provided with an opportunity to read this consent form carefully. All of the questions that I wish to raise concerning this study have been answered. By signing this consent form, I have not waived any of my legal rights or benefits, to which I otherwise would be entitled. My signature indicates that I freely consent to participate in this research study. I will receive a copy of this consent form once I have agreed to participate.

Participant Name, Printed

Participant Signature

Date

Name of Person Conducting Informed Consent Discussion,
Printed

Signature of Person Conducting Informed Consent
Discussion

Date

Principal Investigator Signature (if different from above)

Date

Appendix F
Human Subjects Research Recruitment Documents

Participants Invited
Metabolism Study in Cancer

Are you interested in understanding more about your metabolism?

We are studying patients with cancer who are 40 years and older, not pregnant or breastfeeding, and do not suffer from any serious psychiatric or other medical conditions.

We are looking for volunteers for a study on energy expenditure (energy, or calories, that your body burns). This study will measure metabolism (the breakdown of food and its subsequent change into energy that your body needs) and physiology (the study of the function of body parts and the body as a whole) in patients with cancer. To do this, we use two room calorimeters that can measure a person's energy expenditure and metabolism under different conditions where volunteers can conduct their regular activities.

We will study energy expenditure at rest, for four hours on two different days, under the following conditions:

- 1) 24°C (75°F)
- 2) 27°C (80°F)

We will also ask you to give samples of blood and urine, and to take part in other procedures to measure your heart function, muscle activity, skin temperature, and body composition.

The study is being conducted on the VCUHealth Medical Campus, under the Department of Internal Medicine, Division of Endocrinology, Diabetes, and Metabolism.

Compensation will be provided.

Please contact Dr. Francesco Celi for more information:
Email: Francesco.celi@vcuhealth.org
Phone: 804-828-9696

Sample email contact

Dear Mr./Ms, [include name]

My name s Francesco S. Celi, MD, MHSc. and I am a researcher at the Virginia Commonwealth University. I am contacting you because your provider [include name] at the Massey Cancer Center has indicated that based on your characteristics you could be part in a research on metabolism in patients living with cancer.

Participation in this study is completely voluntary and whether you decide to participate or not doesn't have any impact on the care and treatment that you get from Dr. Dr. [include name] or your other healthcare providers.

Briefly, the study is being conducted at the Clinical Research Service Unit in the 8th floor of North Hospital and will be investigating the energy metabolism of patient with cancer. This could help in designing new interventions to prevent or treat weight loss during cancer treatment.

Please let me know if you are interested in participating and I will be happy to discuss the study at your next visit at VCU, or if you want we can schedule an encounter to discuss the study in detail. There is no compensation for participating in the study.

Please feel free to contacting us by responding to this email or calling us at the following telephone number: 804-828-9696.

Sincerely,

Francesco S. Celi, MD, MHSc.

William G. Blackard Professor of Medicine
Chair, Division of Endocrinology Diabetes and Metabolism
Department of Internal Medicine
Virginia Commonwealth University

1101 East Marshall Street
PO Box 980111
Richmond VA 23298-0111
Phone: 804-828-9696

Sample contact telephone script

Hello Mr/Mrs _____

This is [include name of person contacting] from Virginia Commonwealth University. I am contacting you because your provider [include name] at the Massey Cancer Center has indicated that based on your characteristics you may be interested in a research program on metabolism in patients living with cancer.

Do you have few minutes to talk?

- IF YES, continue.
- If NO: May I call you back at a more convenient time?
 - If YES: ask for date/time that is more convenient.
 - If NO: Ok, I thank you for your time today.

Participation in this study is completely voluntary and whether you decide to participate or not doesn't have any impact on the care and treatment that you get from Dr. [include name] or your other healthcare providers.

This study will be investigating the energy metabolism of patient with cancer. This could help in designing new interventions to prevent or treat weight loss during cancer treatment.

I'd like to send you the consent form which has more information about the study, so you can read it before your clinic visit with Dr. [include name] on __/__/____. Then I can meet with you when you come for that visit, and we can talk more about the study and I can answer any questions you might have. Or if you want we can schedule an encounter to discuss the study in detail. After we talk in person, if you decide you'd like to participate in this study, we will get your signature on the consent form, make sure you meet the criteria for being included in the study and if so, we will get you enrolled. Are you interested in reading the study consent form?

- If YES: Great, what is the best way to send it to you: email, fax, or regular mail? (& confirm email address/fax number/mailling address). I thank you for taking the time to consider participating in this study, and I look forward to seeing you at your clinic visit!
- If NO: Okay, I thank you for taking time to listen to this information.

Please feel free to contacting us at the following telephone number: 804-828-9696 if you have any questions.

The VCU Department of Internal Medicine, Division of Endocrinology, Diabetes, and Metabolism is inviting participants for a research study investigating energy metabolism in cancer. If you or someone you know has cancer and is interested in learning more about this study, please contact Dr. Francesco Celi at francesco.celi@vcuhealth.org for more information.

Appendix G

Human Subjects Research Results Letter

Dear Mr./Mrs./Dr. xxx,

Thank you for participating in our study “Maladaptive adipose tissue activity in cancer” on mm-dd-yyyy. Based on our study, your resting metabolic rates under two different temperatures are shown as follows:

At 24 Celsius: 1.1 kCal/min (this is an example)

At 27 Celsius: 0.9 kCal/min (this is an example)

Resting metabolic rates are the calories you burn per minute, so your total resting energy expenditure at 24°C would be $1.1 \text{ kCal/min} \times 1440 \text{ mins/day} = 1584 \text{ kCal/day}$. This makes up about 60% of your daily energy expenditure, and the other 40% depends on other factors, such as how much you move around during the day.

These results should not be used for therapeutic decision making by your or health care providers.

Thank you again for your participation.
Sincerely,

Francesco S. Celi, MD, MHSc,
William G. Blackard Professor of Medicine
Chair, Division of Endocrinology Diabetes and Metabolism
Department of Internal Medicine
Virginia Commonwealth University

1101 East Marshall Street
PO Box 980111
Sanger Hall, Room 7-007
Richmond VA 23298-0111
Phone: 804-828-9696
Fax 804-828-8389

Appendix H

Human Subjects Research Screening/Case Report Worksheets

Maladaptive adipose tissue activity in cancer Protocol #HM20009089

Subject Screening #: **CAC-**_____

Assign a sequential volunteer ID number that will indicate study identifier, sequential number of screening. Examples: CAC-01, CAC-02

Date: ____/____/____

Informed consent information

Informed consent obtained: Yes ☐ No ☐ Date: ____/____/____ Time ____:____

Informed consent signed/performed by _____

Version of informed consent Version # _____ IRB Approved: ____/____/____

If re-consented:

Version of informed consent Version # _____ IRB Approved: ____/____/____

Withdrew consent on ____/____/____ ☐ Reason: _____

☐ Subject given sufficient time to read and have question answered. A copy of the informed consent given to the subject.

☐ The original signed copy is stored for research; a signed copy of consent given to the subject.

Vital signs

Age (years) _____ Sex: Male ☐ Female ☐

Height (cm) _____ Weight (kg) _____ BMI (kg/m²) _____

Blood pressure (mmHg):

1st reading ____/____

2nd reading ____/____

Average: ____/____

Heart rate (bpm) _____

Temperature (°C) _____

Bio-impedance Analysis

Resistance: _____

Reactance: _____

Screening visit

Version 2 01/19/2019

PI Francesco Celi MD

**Maladaptive adipose tissue activity in cancer
Protocol #HM20009089**

Medical history

Done ☐ Not Done ☐

History of (check yes or no)	YES	NO	N/A	If yes, additional details:
Malignancy				Type, treatments, staging:
Cardiovascular disease				
Hypertension				
Dyslipidemia				
Other				
Diabetes				
Prescription Drugs				ATTACH PRINT OUT OF MEDICATIONS FROM CERNER
Current use of Tobacco				Years, packs per day:
Surgeries				Types, dates:
Pregnant				
Post-menopausal				

Form completed by _____ Date ____/____/____

**Screening visit
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Abbreviated physical exam

Done ☐ Not Done ☐

Organ systems (check normal or not done)	Normal	Not Done	Comments
Neck (thyroid gland size and nodularity)			
Respiratory (sounds)			
Cardiovascular (rate, rhythm, murmur, peripheral pulses)			
Abdominal (tenderness, organomegaly)			
Neurological (focal deficiencies, resting tremor, reflexes)			

M.D. Signature _____ Date ____/____/____

M.D. Print name _____

**Screening visit
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Inclusion /Exclusion Criteria

Inclusion: Exclude if any answers are NO

- ☐ YES ☐ NO ≥ 40 years of age
- ☐ YES ☐ NO Cancer
- ☐ YES ☐ NO PET/CT within past 36 months
- ☐ YES ☐ NO Ability to provide informed consent to participate in study

Exclusion: Exclude if any answers are YES

- ☐ YES ☐ NO Current use of beta-blockers
- ☐ YES ☐ NO Pregnancy or lactation
- ☐ YES ☐ NO Severe claustrophobia
- ☐ YES ☐ NO Remission for cancer

Eligibility

- ☐ The subject **IS** eligible for enrollment base upon meeting all of the Inclusion Criteria and none of the Exclusion Criteria
- ☐ The subject is **NOT** eligible for enrollment base upon not meeting some or all of the Inclusion Criteria and meeting some or all of the Exclusion Criteria

Eligibility Criteria verified by _____ Dated ____/____/____

Comments _____

Assign a sequential volunteer ID number (CAC-01, CAC-02) that will indicate study identifier and sequential number of screening. The suffix "E" will be applied to volunteer who met the inclusion criteria and were enrolled in the study. Example of Enrollment Number: CAC-01E, CAC-02E

Enrollment Number: CAC-_____ E

SSN for payments: _____

Address that subject would like payments mailed: _____

**Screening visit
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Subject #: **CAC-**_____E

Study visit # (circle the visit #): **1(screening)** **2** **3**

Note: Visit 1 (screening) and Visit 2 may occur at same time.

Date: ____/____/____

Procedure	Visit 1 / Screening	Visit 2	Visit 3
After informed consent			
Confirm inclusion/exclusion criteria	X**		
Medical history	X**		
Abbreviated physical examination	X**		
Weight	X		
Vital signs	X		
Bio-impedance analysis	X		
Quality of life (QoL/FACCT) questionnaire	X		
Blood Collection before entering metabolic chamber (12mL)		X	X
Thermal comfort questionnaire before entering metabolic chamber		X	X
Energy recording 4-hour session in metabolic chambers		X	X
Thermal comfort questionnaire after metabolic chamber session completed		X	X
Blood collection after metabolic chamber session completed (12mL)		X	X
Urine collection after metabolic chamber session completed (7mL)		X	X

NOTE: All procedures to be performed by CRSU staff unless marked with "***" which indicates that other protocol investigators may also complete.

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VISIT 1 (SCREENING)

Please ensure that ENTIRE 'screening visit' worksheet is completed - this includes vital signs, bio-impedance analysis, medical history, abbreviated physical exam, inclusion/exclusion criteria, SSN, and address for checks to be mailed (please check and initial when completed):

☐ initials _____

Have subject complete Quality of Life/FAACT Questionnaire (please label with "Subject #") (please check and initial when completed):

☐ initials _____

VISITS 2 & 3

Before entering 'metabolic chambers'

Confirm Consent Obtained: ☐ YES ☐ NO

Confirm 8-hour fast: ☐ YES ☐ NO

Confirm no strenuous activity for 2 days preceding visit: ☐ YES ☐ NO

Ensure subject is wearing light clothing (t-shirt and shorts or light pants, or hospital gown/scrubs).

(please check and initial when completed): ☐ initials _____

Ask subject to void (this will not be collected; only post-session urine will be): (please check and initial when completed): ☐ initials _____

Blood collection (12mL in purple tubes, please label with "HM20009089", Subject # + PRE", & 'Visit # (will be either 2 or 3)') (please check and initial when completed): ☐ initials _____

Have subject complete Thermal Comfort Questionnaire (please label with, Subject # + PRE", & 'Visit # (will be either 2 or 3)') (please check and initial when completed): ☐ initials _____

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Energy expenditure 4-hour recording

Temperature of room for this session (check one): ☐ 24°C ☐ 27°C

Time entered chamber: _____

Time exited chamber: _____

Comments:

After exiting 'metabolic chambers' (visits 2 and 3)

Have subject complete Thermal Comfort Questionnaire (please label with Subject # + POST", & 'Visit # (will be either 2 or 3)') (please check and initial when completed): ☐ initials _____

Blood collection (12mL in purple tubes, please label with "HM20009089", Subject # + POST", & 'Visit # (will be either 2 or 3)') (please check and initial when completed): ☐ initials _____

Urine collection (7mL, please label with "HM20009089", 'Subject #,' & 'Visit # (will be either 2 or 3)') (please check and initial when completed): ☐ initials _____

Provide patient with small snack in CRSU (please check and initial when completed):

☐ initials _____

Provide subject with meal ticket at discharge (please check and initial when completed, and have subject initial on next page):

☐ initials _____

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Meal Ticket Documentation

Visit 2 ____/____/____

- ☐ I have received Meal ticket voucher for my participation in the CAC HM20009089 Study

Participant initials: ____

Visit 3 ____/____/____

- ☐ I have received Meal ticket voucher for my participation in the CAC HM20009089 Study

Participant initials: ____

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Appendix I

Blood/Plasma and Urine Banking Standard Operating Procedure

HM20009089

SOP for blood and urine sample storage

General information:

- If patient has consented to have their blood and/or urine stored for future use, we will receive the following samples at each subject visit:
 - Visit 1 (screening)
 - None
 - Visit 2 (first session in 'metabolic chambers')
 - 4 vials of blood (lavender/purple tops, contain EDTA) *before* subject entered 'chamber'
 - 4 vials of blood (lavender/purple tops, contain EDTA) *after* 'chamber' session completed
 - 1 urine collection vial *after* 'chamber' session completed
 - Visit 3 (second session in 'metabolic chambers')
 - 4 vials of blood (lavender/purple tops, contain EDTA) *before* subject entered 'chamber'
 - 4 vials of blood (lavender/purple tops, contain EDTA) *after* 'chamber' session completed
 - 1 urine collection vial *after* 'chamber' session completed
- Note that Visit 2 and Visit 3 will take place on different days, no more than one month apart.
- CRSU staff should label all containers with the following information:
 - Study number (HM20009089)
 - Subject #
 - Visit # (either 2 or 3)
 - For blood, either 'PRE' or 'POST' to indicated whether blood was collected before or after that day's chamber session.
 - Urine may not have pre/post written because urine is only collected post-session.

PROCEDURES

1. Ensure all samples are collected before proceeding.
 - a. Blood from 'PRE' draw may be stored at room temperature, with vials standing upright, until 'POST' blood and urine are collected.
2. Turn large centrifuge on (if not on already) and set temperature to 4°C to begin cooling.

3. URINE STORING

- a. Transfer 1mL of urine into a 2.0mL corning screw-top cryovial.
- b. Repeat 4 times, for a total of 4mL in 4 vials.
- c. Label all vials with the following information (printed labeled preferred, but written by hand is acceptable):
 - i. Subject # (example: CAC-01-E)
 - ii. Visit # (will be 2 or 3)
 - iii. Urine
 - iv. Date and your initials

4. PLASMA ISOLATION & STORING

- a. Allow time for serum to begin to separate
- b. Move all tubes to large centrifuge (near cell culture hoods) and centrifuge at following settings:
 - i. 4°C
 - ii. 2250 rpm
 - iii. 15 minutes
- c. Transfer 1mL of supernatant (plasma) from each tube and move into a 2.0mL corning screw-top cryovial.
 - i. Repeat for all samples. You should have:
 1. 4 vials with PRE plasma
 2. 4 vials with POST plasma
- d. Label all vials with the following information (printed labeled preferred, but written by hand is acceptable):
 - i. Subject # (example: CAC-01-E)
 - ii. Visit # (will be 2 or 3)
 - iii. PRE or POST plasma (labels by CRSU will tell you which it is for that sample)
 - iv. Date and your initials

5. TRANSFER 12 vials total [urine (4 vials), PRE plasma (4 vials), and POST plasma (4 vials)] into freezer box in -80°C freezer

- a. Ensure boxes are labeled with HM20009089
- b. You may use 2nd shelf "JAV" storage shelves to store these
- c. Please arrange in the following manner (see next page) to ensure each participant's samples stay together for easy use in the future. In this example, gray boxes refer to one subject, green to another, and orange to another. This will allow for ALL samples for 3 SUBJECTS to be kept in one freezer box.

Visit 2 PRE plasma	Visit 2 Urine	Visit 3 POST plasma				Visit 2 PRE plasma	Visit 2 Urine	Visit 3 POST plasma
Visit 2 PRE plasma	Visit 2 Urine	Visit 3 POST plasma	Visit 2 PRE plasma	Visit 2 Urine	Visit 3 POST plasma	Visit 2 PRE plasma	Visit 2 Urine	Visit 3 POST plasma
Visit 2 PRE plasma	Visit 2 Urine	Visit 3 POST plasma	Visit 2 PRE plasma	Visit 2 Urine	Visit 3 POST plasma	Visit 2 PRE plasma	Visit 2 Urine	Visit 3 POST plasma
Visit 2 PRE plasma	Visit 2 Urine	Visit 3 POST plasma	Visit 2 PRE plasma	Visit 2 Urine	Visit 3 POST plasma	Visit 2 PRE plasma	Visit 2 Urine	Visit 3 POST plasma
Visit 2 POST plasma	Visit 3 PRE plasma	Visit 3 Urine	Visit 2 PRE plasma	Visit 2 Urine	Visit 3 POST plasma	Visit 2 POST plasma	Visit 3 PRE plasma	Visit 3 Urine
Visit 2 POST plasma	Visit 3 PRE plasma	Visit 3 Urine	Visit 2 POST plasma	Visit 3 PRE plasma	Visit 3 Urine	Visit 2 POST plasma	Visit 3 PRE plasma	Visit 3 Urine
Visit 2 POST plasma	Visit 3 PRE plasma	Visit 3 Urine	Visit 2 POST plasma	Visit 3 PRE plasma	Visit 3 Urine	Visit 2 POST plasma	Visit 3 PRE plasma	Visit 3 Urine
Visit 2 POST plasma	Visit 3 PRE plasma	Visit 3 Urine	Visit 2 POST plasma	Visit 3 PRE plasma	Visit 3 Urine	Visit 2 POST plasma	Visit 3 PRE plasma	Visit 3 Urine
			Visit 2 POST plasma	Visit 3 PRE plasma	Visit 3 Urine			